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14. ABSTRACT

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. Quiescent leukemia-initiating cells (LICs) are largely resistant to chemotherapy or targeted therapies. The development of new therapeutic approaches that can target LICs will have a profound impact on our ability to eradicate leukemia. We hypothesized that stimulating quiescent LICs to enter the cell cycle will sensitize them to chemotherapy or radiotherapy and improve leukemia treatment. The objective of this application is to further define the role of necdin in regulating LIC quiescence and self-renewal. We have generated two mouse AML models in Necdin null HSCs. We discovered that necdin is not essential for the initiation and progression of AML. We also performed both in vitro and in vivo LIC assays and found that loss of necdin enhances LIC self-renewal. Defining the functions of necdin in normal and leukemic stem cells will provide innovative clinical strategies that are not only useful for eradicating leukemia-initiating cells but will be applicable to the treatment of other human cancers, as well. Successful completion of these studies would be expected to have a potentially important *positive impact* on **military personnel, veterans and their family members**, because an increasing percentage of **Gulf War veterans** are returning from theater with toxin exposure-related AML.

15. SUBJECT TERMS
Acute myeloid leukemia, Leukemia-initiating cell, quiescence, self-renewal, p53, Necdin, chemotherapy, and radiotherapy

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1. Introduction

Acute myeloid leukemia (AML) is a devastating illness with over 13,000 new diagnoses and 10,000 patients dying annually in the United States. AML is the most common acute leukemia in adults. It usually occurs around age 60 with no identifiable cause and it carries a very poor prognosis, with most patients living less than 18 months. The initial treatment of leukemia is designed to achieve a complete remission, meaning that the leukemic cells are not detectable in the bone marrow and normal blood formation has recovered. Leukemia relapse may occur because current therapies eliminate proliferating cells (that constitute the bulk of the disease) but fail to eradicate dormant leukemia-initiating cells (LICs) that can reinitiate the disease after a period of latency (the duration of remission). LICs, and in particular those that are in a dormant state, are resistant to chemotherapy or targeted therapies. The development of new therapeutic approaches that can target LICs will have a profound impact on our ability to eradicate leukemia. Unfortunately, little progress has been made in treating AML over the past 4 decades. Clearly, new treatment strategies are urgently needed. We are turning our attention to the LIC to generate additional knowledge in order to develop therapeutic strategies that can eliminate the largely quiescent leukemia-initiating cells and improve leukemia treatment.

2. Keywords

Acute myeloid leukemia, Leukemia-initiating cell, quiescence, self-renewal, p53, Necdin, chemotherapy, and radiotherapy

3. Accomplishments

What were the major goals of the project?

Recently, we demonstrated that p53 plays a critical role in regulating hematopoietic stem cell (HSC) quiescence and identified necdin as an important p53 target gene in (HSCs). Our objective is to further define the role of necdin in regulating LIC quiescence and self-renewal. We hypothesized that stimulating quiescent LICs to enter the cell cycle will sensitize them to chemotherapy or radiotherapy and improve leukemia treatment. Major goals listed in the Statement of Work (SOW):

Major goals: 1). Define the role of necdin in the initiation of AML. 2). Define the role of necdin in LIC self-renewal. 3). Determine whether downregulating necdin expression in LICs affects their response to chemotherapy or to radiotherapy.

What was accomplished under these goals?

1) **Major activities:** *a) Generating mouse myeloid leukemia models expressing AML-ETO9a or MLL-AF9 in Necdin null HSCs; b) Assess in vitro LIC properties; c) Assess in vivo LIC self-renewal; d) Determine the role of Necdin in leukemia maintenance; and e)Determine the response of Necdin-deficient LICs to chemotherapy and radiotherapy.*

2) **Specific objectives:**
   The specific objectives are to determine the role of necdin in the initiation of acute myelogenous leukemia (AML) and in LIC self-renewal and examine whether lowing of necdin expression

affects the response of LICs to chemotherapy or radiotherapy. Results generated from these objectives will establish Necdin as a novel therapeutic target in human AML.

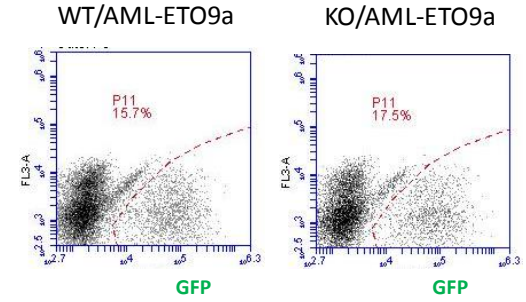

Figure 1. Transduction of wild type (WT) and Necdin null (KO) fetal liver cells with retroviruses expressing AML-ETO9a. Cells within the P11 gate are GFP+.

3) Significant results and key outcomes:

We have achieved all milestones indicated in the Statement of Work (SOW) during the award period. We have generated two mouse AML models in Necdin null HSCs. We also performed both in vitro and in vivo LIC assays to define the role of necdin in the initiation of AML and in LIC self-renewal. We discovered that loss of necdin enhances LIC proliferation and sensitizes LICs to chemotherapy and radiotherapy. Here are significant results and key outcomes:

<u>Generated two mouse models of human AML (MLL-AF9 or AML-ETO9a) in necdin null HSCs:</u>

<u>Retrovirus-mediated transduction of murine fetal liver cells:</u> We infected wild type and Necdin null (KO) fetal liver cells, which contain hematopoietic stem cells (HSCs), with retroviruses expressing GFP (Mock), MLL-AF9 or AML-ETO9a. Robust expression of the GFP was seen 72h post-infection (Figure 1). We have generated two stable fetal liver cell lines expressing either MLL-AF9 or AML-ETO9a.

<u>Generating mouse myeloid leukemia models expressing MLL-AF9 in Necdin null HSCs:</u> MLL-AF9 is a frequently occurring MLL fusion oncogene typically associated with the FAB-M4 or M5 subtypes of human AML. MLL associated leukemias account for the majority of infant leukemia, approximately 10% of adult de novo leukemia and approximately 33% of therapy related acute leukemia with a balanced chromosome translocation. We utilized a mouse model of human acute myeloid leukemia (AML) induced by the MLL-AF9 oncogene to determine the role of Necdin in the initiation and progression of AML. We transduced wild type and Necdin null (KO) fetal liver cells with retroviruses expressing GFP (Mock) or MLL-AF9, as shown in Figure 1, and then transplanted 100,000 GFP^+ cells (CD45.2) into lethally irradiated recipient mice (B6.SJL mice, CD45.1) together with 80,000 normal competitor cells (CD45.1). We monitored leukemia progression in recipient mice by checking GFP+ leukemic cells in the peripheral blood every 4 weeks. As shown in Figure 2, we observed rapid expansion of GFP+ leukemia cells in recipient mice repopulated with both wild type and Necdin null (KO) fetal liver cells

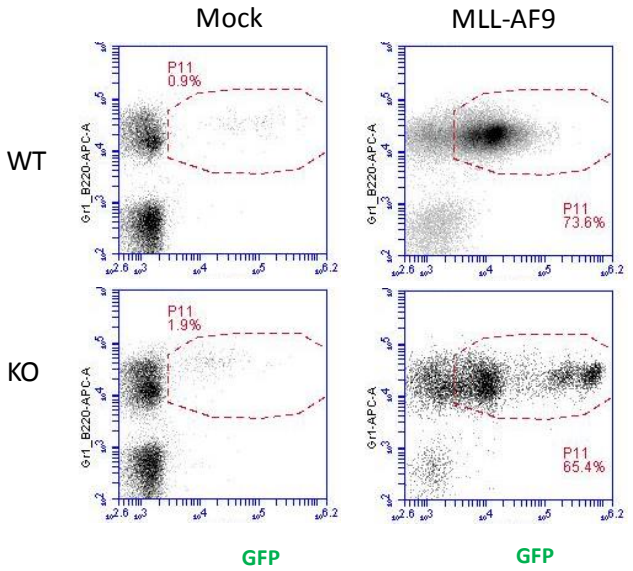

Figure 2. Rapid expansion of GFP+ leukemic cells expressing MLL-AF9 fusion protein in recipient mice 4 weeks following transplantation.

expressing MLL-AF9 compared to mice repopulated with Mock transduced cells, 60-70% GFP+ vs. 1 to 2% GFP+ cells.

Generating mouse myeloid leukemia models expressing AML-ETO9a in Necdin null HSCs: The t(8;21)(q22;q22) translocation is one of the most common genetic abnormalities in acute myeloid leukemia (AML), identified in 15% of all cases of AML, including 40–50% of FAB M2 subtype and rare cases of M0, M1 and M4 subtypes. Expression of AML1-ETO9a leads to rapid development of leukemia in a mouse retroviral transduction–transplantation model. More importantly, coexpression of AML1-ETO and AML1-ETO9a results in the substantially earlier onset of AML and blocks myeloid cell differentiation at a more immature stage. We utilized a mouse model of human acute myeloid leukemia (AML) induced by the AML-ETO9a oncogene to determine the role of Necdin in the initiation and progression of AML. We transduced wild type and Necdin null (KO) fetal liver cells with retroviruses expressing GFP (Mock) or AML-ETO9a, as shown in Figure 1, and then transplanted 100,000 GFP^+ cells (CD45.2) into lethally irradiated recipient mice (B6.SJL mice, CD45.1) together with 80,000 normal competitor cells (CD45.1). We monitored leukemia progression in recipient mice by checking GFP+ leukemic cells in the peripheral blood every 4 weeks. As shown in Figure 3, we observed rapid expansion of GFP+ leukemia cells in recipient mice repopulated with both wild type and Necdin null (KO) fetal liver cells expressing AML-ETO9a compared to mice repopulated with Mock transduced cells.

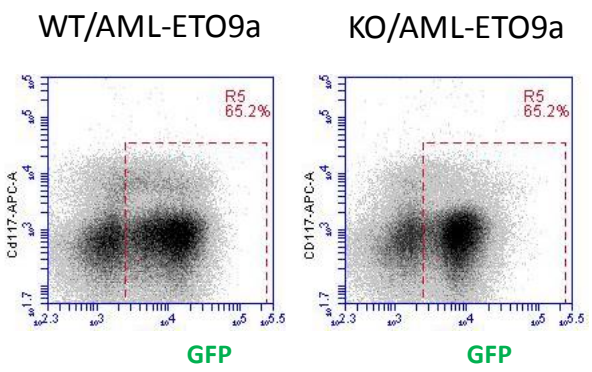

Figure 3. Rapid expansion of GFP+ leukemic cells expressing AML-ETO9a fusion protein in recipient mice 8 weeks following transplantation.

Necdin is not essential for the initiation and progression of AML induced by MLL-AF9 or AML-ETO9a:

In the bone marrow transduction and transplantation model of MLL-AF9, all recipient mice developed AML, with a median latency of 84.5 days. We utilized fetal liver cells and obtained similar results. There is no apparent effect of Necdin deficiency on the survival in primary transplantation. All recipient mice transplanted with wild type or Necdin null mice died 11 weeks following transplantation (Figure 4A). It appears that

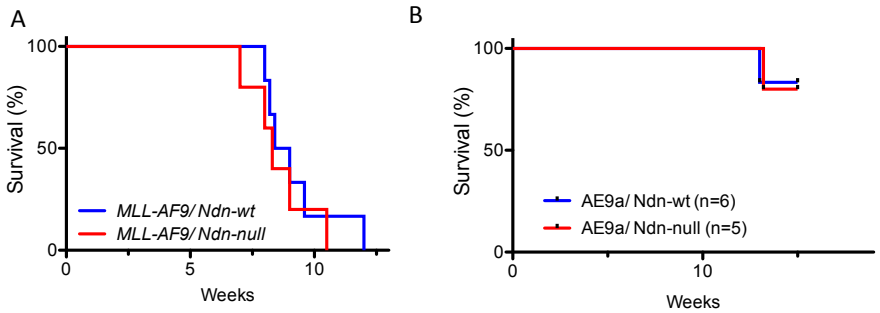

Figure 4. Generating mouse myeloid leukemia models expressing MLL-AF9 or AML1-ETO9a (AE9a) in Necdin null HSCs. (A) Survival curve of animals transplanted with wild type or Necdin null fetal liver cells expressing MLL-AF9 (P=0.2, n=5). (B) Survival curve of animals transplanted with wild type or Necdin null fetal liver cells expressing AML-ETO9a (P=0.1, n=5 or 6)

Necdin is not essential for the initiation and progression of AML induced by MLL-AF9. In the bone marrow transduction and transplantation model of AML1-ETO9a, all recipient mice developed AML, with a median latency of 160 days. We utilized fetal liver cells and obtained similar results. There is no apparent effect of Necdin deficiency on the survival in primary transplantation. Most recipient mice transplanted with wild type or Necdin null mice are still alive 14 weeks following transplantation (Figure 4B).

Loss of necdin decreases LIC self-renewal *in vivo*:

Necdin is highly expressed in long-term hematopoietic stem cells, and we have demonstrated that necdin functions as a rheostat controlling HSC quiescence. Necdin null HSCs are more cycling and more easily exhausted, suggesting that necdin is required for HSC maintenance. Based on our preliminary studies, we hypothesized that necdin is essential for leukemia-initiating cell self-renewal. Serial bone marrow transplantation (BMT) assay is the gold-standard to evaluate the self-renewal potential of both normal HSCs and LICs. We utilized this assay to investigate the function of necdin in LICs *in vivo*. The use of the congenic markers (CD45.2 and CD45.1) will distinguish donor-derived cells from recipient cells, using anti-CD45.2 and anti-CD45.1 antibodies for flow cytometry. The readout of this experiment is the time to the development of an AML-like disease in each "BMT", particularly after the third or fourth BMT.

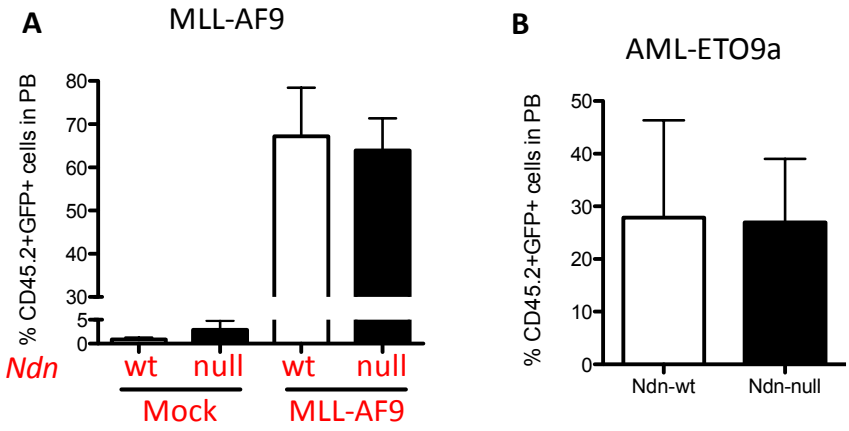

Figure 5. Primary transplantation of fetal liver cells expressing MLL-AF9 (A) or AML-ETO9a (B). Donor-derived GFP+ leukemia cells in the peripheral blood (PB) of primary recipient mice (P=0.2, n=5)

Primary transplantation: To investigate the function of necdin in LICs *in vivo*, we transplanted wild type or necdin null fetal liver cells (CD45.2) transduced with AML-ETO9a or MLL-AF9 into lethally irradiated recipient mice (B6.SJL, CD45.1; 30 mice in total, 5 mice per group). In primary transplantation, we observed no difference in donor-derived GFP+ cells in mice repopulated with wild type or necdin null HSCs expressing either MLL-AF9 or AML-ETO9a (Figure 5). We sacrificed the primary recipient mice at 4 weeks or 8 weeks following transplantation, for MLL-AF9 and AML-ETO group respectively, and harvested GFP+ bone marrow cells for secondary transplantation.

Secondary transplantation: We utilized Limiting-dilution transplantation assays to enumerate LIC numbers *in vivo*. We transplanted 3 $x10^6$ or 3 $x10^5$ GFP+ AML1-ETO+ bone marrow cells (CD45.2) isolated from the primary recipient mice into lethally irradiated recipient mice (B6.SJL, CD45.1; 20 mice in total, 5 mice per group). We monitored engraftment of GFP+ leukemia cells in peripheral blood by flow cytometry every 4 weeks after transplantation. We found that necdin-null LICs showed decreased engraftment using both concentrations of donor leukemia cells 16 weeks post transplantation

(Figure 6). We obtained similar results in LICs expressing MLL-AF9. These data demonstrate that necdin is essential for LIC self-renewal.

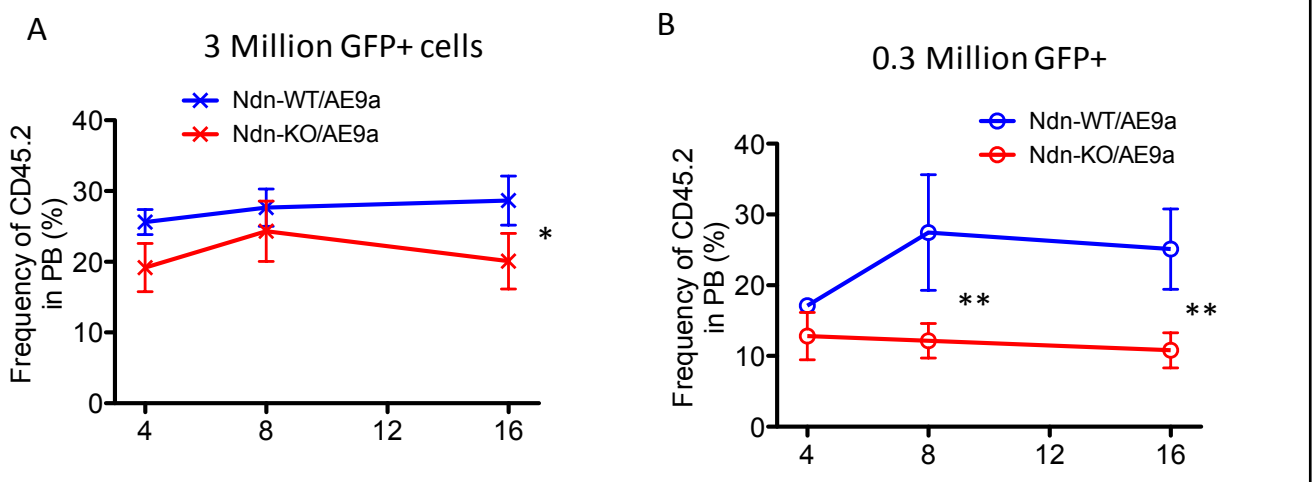

Figure 6. Secondary transplantation of LICs expressing AML-ETO9a. Donor-derived GFP+ leukemia cells in the peripheral blood (PB) of the secondary recipient mice (*P<0.05, **P<0.01, n=5)

Necdin is essential for the maintenance of AML induced by MLL-AF9:

We found that necdin is not essential for the initiation and progression of leukemia induced by MLL-AF9 and AML1-ETO8a (Figure 4). Given that we found that necdin is essential for LIC self-renewal (Figure 6), we predicted that necdin is important for leukemia maintenance. We transplanted 3 x 10^6 GFP+ MLL-AF9+ bone marrow cells (CD45.2) isolated from the primary recipient mice into lethally irradiated recipient mice (B6.SJL, CD45.1). We monitored leukemia development and survival of recipient mice. While it takes 10 weeks for MLL-AF9 to induce leukemia in the primary recipient mice (Figure 4A), it only takes 3 weeks develop leukemia in secondary recipient (Figure 7). We found that necdin-deficiency significantly delayed leukemia onset (Figure 7), demonstrating that necdin is essential for leukemia maintenance. Given that it takes at least 4 to 6 months for AML1-ETO to induce leukemia in secondary recipient, we expect that necdin will be important for leukemia maintenance induced by AML1-ETO.

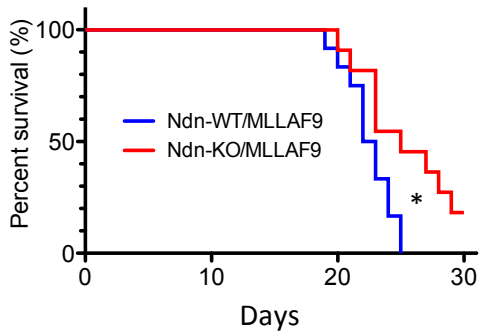

Figure 7. Survival curve of animals transplanted with wild type or Necdin null fetal liver cells expressing MLL-AF9 (*P<0.05, n=7)

Loss of necdin enhances LIC proliferation:

We found that p53 promotes HSC quiescence, and in its absence, HSCs more easily enter the cell cycle. Furthermore, we identified Necdin (Ndn) as a novel p53 target genes in HSCs. As quiescence has been

shown to protect stem cells from DNA damage, we analyzed the cell cycle status of necdin null hematopoietic stem cells using Ki67 and Hoechst staining, and found that necdin null HSCs are less quiescent than wild type stem cells. To determine the effect of necdin loss on LIC proliferation, we performed liquid culture assays. We cultured wild type and necdin null fetal liver cells expressing AML-ETO9a (AE9a) or MLL-AF9 in medium containing hematopoietic cytokines for 7 days and then counted cell number by using flow cytometry. While expression of MLL-AF9 has no effect on the proliferation of both wild type and necdin null HSCs, expression of AML-ETO9a enhances the proliferation of necdin null HSCs compared to wild type HSCs (Figure 8). These data indicate that Necdin loss enhances the proliferation of LICs expressing AML-ETO9a, but not MLL-AF9, in liquid culture assays.

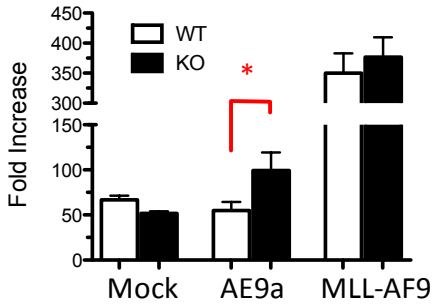

Figure 8. Liquid culture of LICs expressing MLL-AF9 or AML-ETO9a (p=ressing MLL-AF9 or AML-ETO9a (*P<0.05, n=3)0.05, n=3).

We then examined proliferation potential of LICs using the serial replating assays. The serial replating potential of LICs correlates with their *in vivo* proliferation capability. Fetal liver cells from wild type and Necdin null mice were transduced with MLL-AF9 or AML-ETO9a and serially replated in the semisolid medium. While necdin null LICs show decreased colony formation in week one, Necdin null LICs expressing MLL-AF9 or AML-ETO9a show enhanced colony formation than that of wild type cells (Figure 9) by week 4, indicating that loss of necdin enhances LIC proliferation.

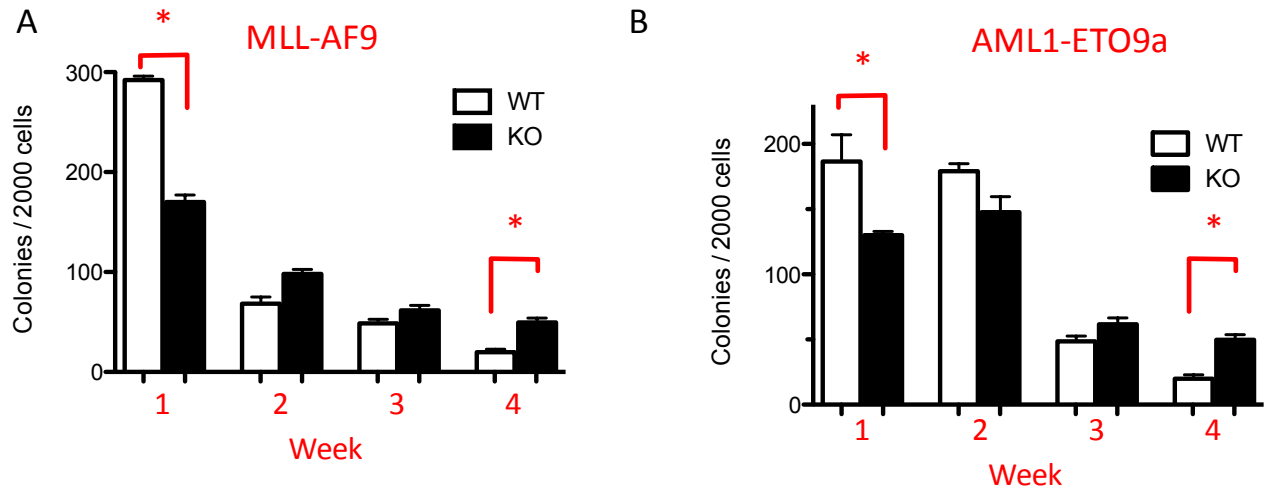

Figure 9. Evaluating LIC porliferation in vitro by using the serial replating assays. Necdin null LICs expressing MLL-AF9 (A) or AML-ETO9a (B) show enhanced replating potential (*P<0.05, n=3).

Loss of necdin sensitizes LICs to chemotherapy and radiotherapy:

Given that we found that loss of necdin enhances LIC proliferation (Figures 8 and 9), we predict that necdin –deficient LICs will be sensitive to chemotherapy or radiotherapy. That was in deed the case. We treated WT and necdin null LICs expressing MLL-AF9 with different concentration of chemotherapy drug cytarabine (Ara-C) and monitored cell viability by cell counting. 48 hours after Ara-C treatment,

we found that necdin null LICs expressing MLL-AF9 were sensitive to Ara-C treatment in a dosage-dependent manner (Figure 10), manifested by decreased viability. We also found necdin null LICs expressing MLL-AF9 are sensitive to low dose irradiation (2 Gy) compare to WT LICs. We transplanted WT and necdin null LICs into recipient mice to determine whether necdin null LICs expressing MLL-AF9 are sensitive to chemotherapy or radiotherapy *in vivo*. As shown in Figure 7, leukemia developed very fast in secondary recipient and most recipient mice died within 3 to 4 weeks following transplantation, making it difficult to treat leukemic mice. We decreased the dose of donor leukemia cell and then did the transplantation. These *in vivo* chemotherapy and radiotherapy experiments are ongoing. We predict that lowing necdin level in LICs will sensitize them to chemotherapy or radiotherapy, manifested by decreased leukemia burden and extended survival.

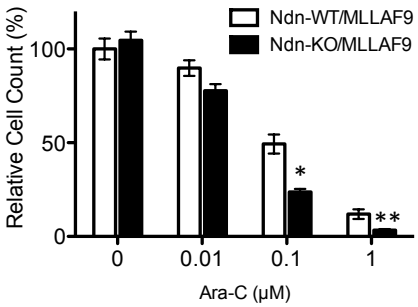

Figure 10. Necdin-deficient LICs are sensitive to chemotherapy treatment. WT and necdin null LICs expressing MLL-AF9 were treated with different concentration of chemotherapy drug cytarabine (Ara-C). The viability of LICs was measured by cell counting (*P<0.05, **P<0.01, n=3).

Human leukemia patients with MLL-AF9 are resistant to chemotherapy, our data demonstrate that necdin is a viable therapeutic target in human AML and pharmacological inhibition of necdin will eradicate leukemia-initiating cells and improve leukemia treatment.

4) Other achievements:

Nothing to report.

What opportunities for training and professional development has the project provided?

This award has significantly enhanced Dr. Liu's career development. Dr. Liu published 9 peer-reviewed scientific papers and had three manuscripts that are in revision. In addition, Dr. Liu submitted 15 abstracts to national and international scientific conferences. Based on data generated from this award, Dr. Liu obtained four external funding, including a Scholar Award from the St. Baldrick's foundation ($330,000), a New Investigator Award the Elsa Pardee Foundation ($125,000), a New Investigator Award from American Cancer Society, and a research grant from Alex's Lemonade Stand Foundation. Dr. Liu is also a Co-investigator on Dr. Yoshimoto's NIH R56 grant. Dr. Liu served as grant reviewers for national and international scientific organizations, including New York State Department of Health, National Institutes of Health (NIH), St. Baldrick's Foundation, and the National Natural Science Foundation of China (NSFC). Moreover, Dr. Liu is on the editorial boards of 4 scientific journals, including International Journal of Neonatal Science and Therapeutics, Journal of Stem Cell Research and Transplantation, Journal of Stem Cells and Translational Investigation, and Pediatrics and Neonatal Biology. Dr. Liu applied for promotion to Associate Professor with Tenure in May 2015. The Department of Pediatrics and Indiana University School of Medicine had approved his application. Dr. Liu will be an Associate Professor with Tenure on July 1, 2016. Overall, this award facilitated the transition of Dr. Liu to an independent investigator funded by extramural support.

How were the results disseminated to communities of interest?

The results from this award were disseminated to communities of interest through publications, meeting presentations, as well as invited presentations. Please see lists of publications, abstracts, and invited presentations in **Section 6 Products**.

What do you plan to do during the next reporting period to accomplish the goals?

Nothing to report.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Quiescent leukemia-initiating cells (LICs) appear to be responsible for resistance to conventional chemotherapy or radiotherapy, providing an explanation for why conventional cancer therapies may reduce tumor burden, but not result in cures. We found that loss of necdin sensitizes LICs to chemotherapy or radiotherapy and enhances leukemic cell clearance. Therefore, Necdin will be a *novel therapeutic target* in AML treatment. The development of a new therapeutic approach that can improve leukemia treatment in experimental animals would help to establish a strong scientific framework for eventual clinical trials in humans. Therefore, our studies will have a potentially important *positive impact* on **military personnel, veterans and their family members**, because an increasing percentage of **Gulf War veterans** are returning from theater with toxin exposure-related AML.

What was the impact on other disciplines?

Defining the functions of necdin in normal and leukemic stem cells will help provide innovative clinical strategies that are not only useful for eradicating leukemia-initiating cells but will be applicable to the treatment of other human cancers, as well.

What was the impact on technology transfer?

The proposed work will facilitate the development and clinical application of *Necdin inhibitor* in treating military personnel, veterans and their dependents with leukemia and improve their quality of life.

What was the impact on society beyond science and technology?

Nothing to report.

5. Changes/Problems

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to report.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report.

6. Products

Publications, conference papers, and presentations:

Journal Publications:

1. Asai T, **Liu Y,** and Nimer SD. Necdin, a p53 target, in normal and cancer stem cells. *Oncotarget.* 4(6):806-7, 2013. PMCID: PMC3757235
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Invited presentations:

2013 **Yan Liu, Ph.D.,** "Bmi1 regulates ribosome biogenesis during erythroid differentiation". The 55th American Society of Hematology (ASH) Conference, New Orleans, LA.

2014 **Yan Liu, Ph.D.,** "BMI1 promotes erythropoiesis through regulating ribosome biogenesis". The 13th Diamond Blackfan Anemia International Consensus Conference, Atlanta, GA.

2014 **Yan Liu, Ph.D.,** "PRL2 phosphatase in normal and malignant hematopoiesis". Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences, Tianjin, China

2014 **Yan Liu, Ph.D.,** "Polycomb group protein Bmi1 regulates hematopoietic stem cell self-renewal and differentiation". National Institute of Biological Sciences, Beijing, China

2015 **Yan Liu, Ph.D.,** "Mutant p53 enhances hematopoietic stem cell self-renewal through regulating epigenetic regulators". Keystone symposium: Hematological Malignancies. Keystone, CO.

2015 **Yan Liu, Ph.D.,** "PRC1 complex enhances hematopoietic stem cell self-renewal through inhibiting Wnt signaling". Midwest Blood Club 2015. French Lick, Indiana.

2015 **Yan Liu, Ph.D.,** "PRC1 complex enhances hematopoietic stem cell self-renewal through inhibiting Wnt signaling". The 57th American Society of Hematology (ASH) Conference, Orlando, FL.

Other Products:

Development of cell lines:

Necdin null hematopoietic stem cells expressing oncoprotein AML-ETO9a
Necdin null hematopoietic stem cells expressing oncoprotein MLL-AF9

Development of animal models:

Mouse model of human acute myeloid leukemia expressing oncoprotein AML-ETO9a
Mouse model of human acute myeloid leukemia expressing oncoprotein MLL-AF9

Funding applied for based on work supported by this award:

St. Baldrick's Foundation Scholar Award (PI: Liu Y) 07/01/14 – 06/30/17
St. Baldrick's Foundation
Targeting PRL2 phosphatase in T cell acute lymphoblastic leukemia
Role: Principal Investigator

Pardee Foundation Research Grant (PI: Liu Y) 01/01/14 – 12/31/14
Elsa U. Pardee Foundation
Targeting PRL2 in Acute Myeloid Leukemia
Role: Principal Investigator

ACS Institutional Research Grant (PI: Liu Y) 06/01/13 – 5/31/14 American Cancer Society and Indiana University Simon Cancer Center (IUSCC)
The role of PRL2 Phosphatase in Acute Myeloid Leukemia
Role: Principal Investigator

Alex's Lemonade Stand Foundation Grant (PI: Liu Y) 09/01/14 – 08/31/15
Alex's Lemonade Stand Foundation
Targeting PRL2 phosphatase in Pediatric acute myeloid leukemia
Role: Principal Investigator

R56AI110831 (PI: Yoshimoto) 3/10-15-2/29/16
NIH/NIAID
Embryonic origin and Self-renewal of B-1a cells
The goal of this study is to identify the origin of B1 cells during development and investigate the mechanisms of B-a cell self-renewal.
Role: Co-Investigator

IUSCC Pilot Project (PI: Liu Y) 05/01/15 – 04/30/16
Indiana University Simon Cancer Center (IUSCC)
Targeting mutant p53 to improve leukemia treatment
Role: Principal Investigator

Wells Center Seed Fund Grant (PI: Liu Y) 06/01/15 – 12/31/15
Wells Center for Pediatric Research
PRL2 phosphatase as a novel therapeutic target in Acute Myeloid Leukemia
Role: Principal Investigator

Positions and honors:

2013 to present	**Yan Liu, Ph.D.,** Member, NYSTEM Study Section, New York State Department of Health and the Empire State Stem Cell Board
2014 to present	**Yan Liu, Ph.D.,** Member, the Early Career Reviewer (ECR) program at the Center for Scientific Review (CSR), National Institutes of Health
2014 to present	**Yan Liu, Ph.D.,** Foreign Expert (Member), Hematology Study Section, National Natural Science Foundation of China (NSFC), China.
2014 to present	**Yan Liu, Ph.D.,** Member, Career Development Award (CDA) Program, International Human Frontier Science Program Organization (HFSP), France
2015 to present	**Yan Liu, Ph.D.,** Member of Cancer Biology Study Section, the St. Baldrick's Foundation.
2014 to 2015	**Yan Liu, Ph.D.,** Abstract reviewer, 56th and 57th Annual Meeting of the American Society of Hematology (ASH), the session of Malignant Stem and Progenitor Cells.

Editorial Board:

2014 to present	**Yan Liu, Ph.D.,** Editor, International Journal of Neonatal Science and Therapeutics
2014 to present	**Yan Liu, Ph.D.,** Editor, Journal of Stem Cell Research and Transplantation
2014 to present	**Yan Liu, Ph.D.,** Editor, Stem Cells Research, Development and Therapy

2014 to present **Yan Liu, Ph.D.,** Editor, Journal of Stem Cells and Translational Investigation

2014 to present **Yan Liu, Ph.D.,** Editor, Pediatrics and Neonatal Biology

7. Participants & other Collaborating Organizations

What individuals have worked on the project?

Name:	*Yan Liu, Ph.D.*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*0000-0003-4878-9111*
Nearest person month worked:	*5*
Contribution to Project:	*Dr. Liu has supervised the conduct of the experiments, analyzed data, developed hypotheses, planed future experiments. Dr. Liu will write, revise, and submit manuscripts describing the findings from this project.*
Funding Support:	*This DoD Career Development Award and St. Baldrick's Foundation.*

Name:	*Michihiro Kobayashi, M.D. & Ph.D.*
Project Role:	*Postdoctoral Fellow*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-6065-6026*
Nearest person month worked:	*24*
Contribution to Project:	Dr. Kobayashi has generated mouse leukemia models, conducted the bone marrow transplant studies, and performed flow cytometric analysis of cells isolated from leukemic mice. Dr. Kobayashi has performed treatment of leukemia cells with chemotherapy drugs. He has designed and conducted all the experiments listed in this proposal. He will write and prepare manuscripts for submission.
Funding Support:	

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. Special reporting Requirements

Nothing to report.

9. Appendices

A: 9 published Papers

B: 15 Abstracts

C: Yan Liu, CV

D: Federal Financial Report

Necdin, a p53 target gene, in stem cells

Takashi Asai, Yan Liu, and Stephen D Nimer

Necdin, a member of the melanoma antigen gene (MAGE) family, originally identified to suppress cell proliferation in post-mitotic neurons, facilitates the entry of the cell into *cell cycle, functions like retinoblastoma (Rb) protein, and acts as a transcriptional repressor that recognizes* guanosine (G)-rich DNA sequences. Necdin also binds to p53 and inhibits p53-dependent apoptosis in these cells, demonstrating its function as an endogenous anti-mitotic and anti-apoptotic protein in post-mitotic neurons [1]. The *necdin* gene is located on chromosome 15 in human and its expression is controlled through genomic imprinting; it is one of the genes that have been associated with the human Prader-Willi syndrome, a genetic neuro-behavioral disorder [2]. Necdin is expressed in some stem or undifferentiated progenitor cells such as mesoangioblast stem cells [3], vessel-associated stem cells [4], adipocyte progenitor cells [5, 6], and hematopoietic stem cells (HSCs) [7], yet the function of necdin in these cells remains unclear.

While highly expressed in long-term HSCs, transcript profiling of hematopoietic stem/progenitor cells (HSPCs) isolated from wild-type and p53 null mice identified *necdin* as being a p53 target gene [7]. We then demonstrated using chromatin immunoprecipitation assays that the *necdin* promoter contains a p53 binding site and that necdin is a direct transcriptional activator of p53 in HSPCs. Lentivirus-mediated knock down or over-expression of necdin resulted in decreased or increased HSPC quiescence, respectively [7].

We recently clarified the function of necdin in HSCs and HSPCs using necdin null mice [8]. Necdin null mice show the same phenotype as the patients with Prader-Willi syndrome and because they die perinatally, we transplanted necdin null fetal liver cells into lethally irradiated wild type recipients to reconstitute their bone marrow function and analyzed the HSC intrinsic function of necdin in adult hematopoiesis. Necdin-null HSCs have normal self-renewal capacity after serial transplantation, while we found that necdin-null HSCs are less quiescent and more proliferative than normal, suggesting that necdin has the similar function as p53 in promoting HSC quiescence in the steady state. We then demonstrated that necdin-null HSCs are highly sensitive to genotoxic stress, irradiation or chemotherapy, with increased apoptosis via both cell-cycle-dependent and cell-cycle independent mechanisms. We further found that the function of necdin in hematopoiesis in response to genotoxic stress depends on Gas2L3, a novel member of the Gas2 family, using lentivirus-mediated knock down or over-expression of Gas2L3 in HSPCs [8]. Therefore, necdin appears to function as a molecular switch in adult hematopoiesis: to promote HSC quiescence in a p53-like manner in the steady state, but to suppress p53-dependent apoptosis under the stress conditions.

Acute leukemia is thought to begin in rare leukemia-initiating cells (LICs) that maintain or re-acquire the capacity for indefinite self-renewal through accumulated mutations and/or epigenetic changes. LICs, particularly those in a quiescent state, are thought to be resistant to chemotherapy or targeted therapies, and it is hypothesized that leukemia relapse may occur because current therapies just eliminate proliferating cells, but fail to eradicate quiescent LICs that can reinitiate the disease once a patient is in remission. Therefore, the development of new therapeutic approaches that can target LICs may have an impact on our ability to eradicate acute leukemia. Considering that necdin-null HSCs are more proliferative and show enhanced sensitivity to chemotherapy, or irradiation, targeting necdin may provide a possible therapeutic approach to eliminating quiescent LICs.

The functions of necdin in other stem or progenitor cells have recently been reported. Necdin is highly expressed in adipocyte progenitor cells and knockdown of necdin accelerates adipocyte differentiation and proliferation [5]. Similarly, necdin null mice show enhanced adipocyte progenitor cell proliferation and increased white adipocyte expansion [6]. These functions of necdin in adipocyte progenitor cells are similar to those seen in HSPCs. Necdin is also significantly expressed in vessel-associated stem cells, such as mesoangioblasts (MABs); in this system, the overexpression of necdin in MABs enhances myogenic differentiation and improves cell survival after the toxin administration [4]. Thus, the functions of necdin in stem or progenitor cells vary depending on the cell context.

Further studies are needed to clarify the function of necdin in various normal and cancer stem cell populations. However, based on enhanced chemosensitivity, and radiosensitivity, identified in necdin null HSCs, necdin may be a promising therapeutic target to enhance the killing of dormant, cancer stem cells.

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Cell Reports
Article

PRMT4 Blocks Myeloid Differentiation by Assembling a Methyl-RUNX1-Dependent Repressor Complex

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SUMMARY

Defining the role of epigenetic regulators in hematopoiesis has become critically important, because recurrent mutations or aberrant expression of these genes has been identified in both myeloid and lymphoid hematological malignancies. We found that PRMT4, a type I arginine methyltransferase whose function in normal and malignant hematopoiesis is unknown, is overexpressed in acute myelogenous leukemia patient samples. Overexpression of PRMT4 blocks the myeloid differentiation of human stem/progenitor cells (HSPCs), whereas its knockdown is sufficient to induce myeloid differentiation of HSPCs. We demonstrated that PRMT4 represses the expression of miR-223 in HSPCs via the methylation of RUNX1, which triggers the assembly of a multiprotein repressor complex that includes DPF2. As part of the feedback loop, PRMT4 expression is repressed posttranscriptionally by miR-223. Depletion of PRMT4 results in differentiation of myeloid leukemia cells in vitro and their decreased proliferation in vivo. Thus, targeting PRMT4 holds potential as a novel therapy for acute myelogenous leukemia.

INTRODUCTION

Arginine methylation is a common posttranslational modification that regulates the function of a wide range of proteins. There are ten members of the protein arginine methyltransferase (PRMT) family, eight of which catalyze the formation of either asymmetric dimethylarginine (the type I enzymes) or symmetric dimethylarginine (the type II enzymes) (Bedford and Clarke, 2009). The type I protein arginine methyltransferase 4 (PRMT4), also called coactivator-associated arginine methyltransferase 1 (CARM1), functions as a coactivator of nuclear hormone receptors as well as other transcription factors including p53 (An et al., 2004), NF-kappa B (Covic et al., 2005), β-catenin (Koh et al., 2002), and Mef2c (Chen et al., 2002). PRMT4 can methylate the transcriptional coactivator p300 (Xu et al., 2001; Lee et al., 2011) and several histone substrates, in particular, H3R17 and H3R26 (Daujat et al., 2002; Schurter et al., 2001). PRMT4 plays an important role in a number of biological processes including muscle cell differentiation (Chen et al., 2002), T cell development (Kim et al., 2004), and adipocyte differentiation (Yadav et al., 2008). PRMT4 maintains embryonic stem cell (ESC) pluripotency and inhibits ESC differentiation (Torres-Padilla et al., 2007; Wu et al., 2009). Although other members of the PRMT family have been implicated in hematopoiesis and acute leukemia (Zhao et al., 2008; Cheung et al., 2007; Liu et al., 2011), little is known about the role of PRMT4 in normal or malignant hematopoiesis.

RUNX1 (also known as AML1) is a transcription factor that binds to a consensus binding sequence (CBS) PyGpyGGTPy (Py = pyrimidine) in the regulatory regions of promoters and enhancers of genes that play important roles in hematopoiesis. RUNX1 knockout mice die between embryonic day (E) 11.5 and E13.5 with a complete lack of fetal liver (i.e., definitive) hematopoiesis (Okuda et al., 1996), whereas conditional deletion of *RUNX1* in adult mice results in profound lineage-specific abnormalities, including a block in lymphoid development and

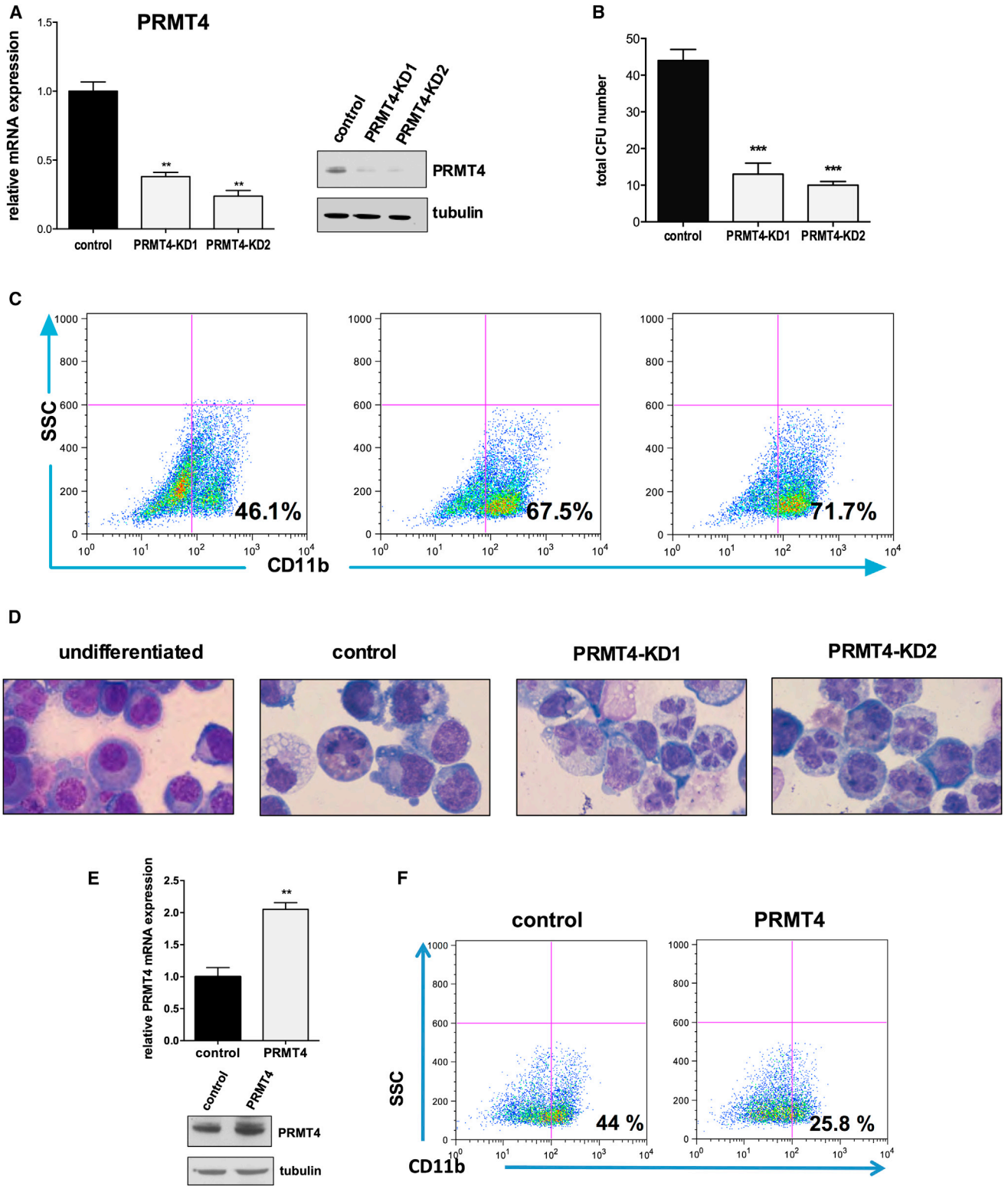

Figure 1. PRMT4 Regulates Myeloid Differentiation of HSPCs

(A) Efficient knockdown of PRMT4. Human CB CD34$^+$ cells were transduced with lentiviruses expressing a control (scrambled) shRNA or one of two shRNAs directed against PRMT4. GFP-positive cells were sorted 3 days after transfection and collected to perform qRT-PCR and western blot analyses. qRT-PCR data represent the mean ± SD of the three independent experiments. **p < 0.01 by Student's t test. Tubulin served as the loading control.

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reduced megakaryocytic production, with little effect on adult hematopoietic stem cells (HSCs). *RUNX1* is one of the most frequently altered genes in acute leukemia, either by chromosomal translocations such as the t(8;21) (Blyth et al., 2005) or by point mutations or deletions that occur in 4%–10% of patients with sporadic or therapy-related myelodysplastic syndrome and acute myelogenous leukemia (AML) (Osato, 2004). Furthermore, *RUNX1* point mutations are found in affected individuals with the inherited FPD/AML syndrome (familial platelet disorder with propensity to AML) (Song et al., 1999). Posttranslational modifications, including ubiquitination, phosphorylation, acetylation, and methylation fine-tune RUNX1 function (Wang et al., 2009); for instance, arginine methylation of an RTAMR motif in RUNX1 by PRMT1 abrogates SIN3A binding, thereby potentiating RUNX1-dependent transcriptional activation of its target genes (Zhao et al., 2008). Similarly, microRNAs such as miR-17-5p, miR-20a, and miR-106a can regulate RUNX1 protein expression and thereby control aspects of hematopoietic cell differentiation (Fontana et al., 2007).

The myeloid-specific microRNA-223 (miR-223) has been shown to affect granulocytic differentiation. Loss of miR-223 impairs granulocytic maturation (Johnnidis et al., 2008), whereas miR-223 overexpression promotes myeloid differentiation (Fazi et al., 2005). miR-223 expression has been shown to be transcriptionally regulated by NF-IA (Fazi et al., 2005), by C/EBPs and PU.1 (Fukao et al., 2007) and by E2F1 (Pulikkan et al., 2010). Fazi et al. reported that the AML1-ETO fusion protein represses miR-223 expression by binding to a RUNX CBS located upstream of the pre-miR-223 (Fazi et al., 2007). They, and others, have found that miR-223 expression is downregulated in AML patient samples (Fazi et al., 2007; Pulikkan et al., 2010; Eyholzer et al., 2010).

In this study, we have identified PRMT4 as a regulator of the myeloid differentiation process in stem/progenitor human hematopoietic cells (HSPCs). PRMT4 forms a regulatory loop with miR-223, to reciprocally suppress the expression of each other during myeloid differentiation. We also show that RUNX1 is a substrate of PRMT4 and that methylation of RUNX1 by PRMT4 (on arginine 223 [R223]) leads to the recruitment of a DPF2-containing repressor complex that binds critical miR-223 transcriptional regulatory elements (that are distinct from the element identified by Fazi et al., 2007) and represses miR-223 expression. A decrease in PRMT4 expression is critical for normal myeloid differentiation, but this normal event can be subverted by malignant hematopoietic cell transformation. Thus, targeting PRMT4 represents a potential therapeutic approach to promote the differentiation of AML blast cells.

RESULTS

PRMT4 Regulates Myeloid Differentiation

To examine its function in hematopoiesis, we knocked down PRMT4 in human cord blood (CB)-derived, $CD34^+$ hematopoietic stem/progenitor cells (HSPCs), using lentiviral vectors that express GFP and short hairpin RNAs (shRNAs) directed against PRMT4. We assayed the extent of PRMT4 knockdown (KD) in the GFP-positive transduced cells and found a 70%–80% decrease in PRMT4 expression for the KD1 and KD2 short hairpin RNAs, respectively (Figure 1A). The PRMT4-KD cells generated far fewer CFUs when plated in methylcellulose (Figure 1B) and showed enhanced myeloid differentiation after 7 days in myeloid-promoting liquid culture (which contains stem cell factor [SCF], FLT-3 ligand, interleukin [IL]-3, IL-6, granulocyte-macrophage colony stimulating factor [GM-CSF], and granulocyte colony stimulating factor [G-CSF]) with 60%–70% of the KD cells being CD11b positive and CD13 positive, versus 40% (and 14%) of the control cells being CD11b (or CD13) positive (Figures 1C and S1B). Consistent with the immunophenotypic evidence, morphologic evidence also showed more mature myeloid cells following PRMT4 KD (Figure 1D), with the KD cells showing a condensed nucleus and clear nuclear lobulation. In addition, PRMT4 KD mildly impaired erythroid differentiation under erythroid-promoting culture conditions (SCF and erythropoietin) (Figure S1E). Consistent with its effect on CFU generation, a decrease in the numbers and size of the cobblestone areas was seen at week 5, reflecting impaired HSPC self-renewal when PRMT4 was knocked down (Figure S1D).

We next examined whether PRMT4 overexpression blocks myeloid differentiation. Indeed, we found a marked reduction in CD11b-positive cells generated from PRMT4-overexpressing $CD34^+$ cells (compared to the control cells) after 7 days in myeloid differentiation promoting cultures (Figure 1E). Thus, PRMT4 appears to be an important negative regulator of normal myeloid differentiation.

PRMT4 Is Regulated Posttranscriptionally by miR-223 during Myeloid Differentiation

Given the prominent effect of PRMT4 on myeloid differentiation, we assessed changes in PRMT4 expression during normal in vitro HSPC differentiation. We cultured human CB $CD34^+$ cells

(B) Downregulation of PRMT4 decreases CFU formation. 10^4 of the control or PRMT4 knockdown cells were plated in methylcellulose. The total number of colony forming units (CFUs) was scored 2 weeks after the plating. The data represent the mean ± SD of the three independent experiments. ***$p < 0.001$ by Student's t test.

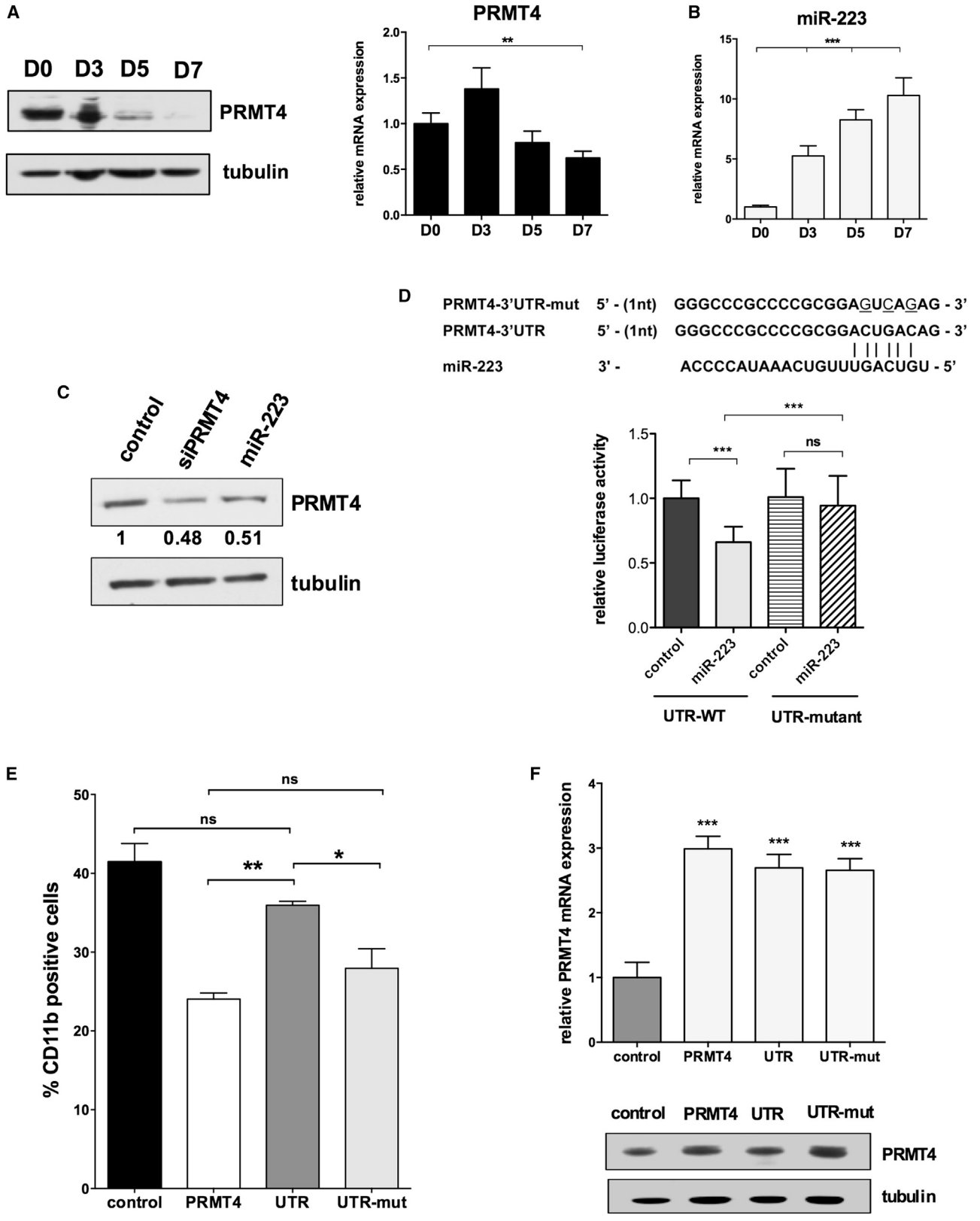
(C) Downregulation of PRMT4 promotes the myeloid differentiation of HSPCs. GFP^+ $CD34^+$ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Myeloid differentiation was determined by fluorescence-activated cell sorting (FACS) analysis of CD11b expression.

(D) Downregulation of PRMT4 promotes the myeloid differentiation of HSPCs. Cellular morphology was evaluated after 7 days in myeloid-promoting cytokine containing medium. Cells growing in basic culture were used as the control for myeloid differentiation.

(E) Overexpression of PRMT4 was demonstrated at the mRNA and protein levels. Human CB $CD34^+$ cells were transduced with retroviruses expressing either control (GFP alone) or GFP and HA-PRMT4. GFP^+ cells were sorted after 3 days of transfection and collected to perform qRT-PCR and western blot analyses. qRT-PCR data represent the mean ± SD of the three independent experiments. **$p < 0.01$ by Student's t test.

(F) Overexpression of PRMT4 blocks the myeloid differentiation of HSPCs. GFP^+ $CD34^+$ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD11b expression.

See also Figure S1.



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in myeloid-promoting liquid culture and observed a significant decrease in PRMT4 protein levels over a 7 day period (Figure 2A). PRMT4 mRNA levels varied only slightly during this process, suggesting that PRMT4 is being regulated posttranscriptionally. MicroRNA target prediction programs (TargetScan release 5.2 [Figure S2A] and PITA) suggested that PRMT4 is a potential target of several microRNAs, including miR-223, a myeloid-specific microRNA. Interestingly, a seed sequence for miR-223 is found in the 3′ UTR region of PRMT4, which is located adjacent to the stop codon of the PRMT4 open reading frame (ORF) (16–22 nt from the stop codon); this location could confer a strong, translational regulatory effect (Grimson et al., 2007; Eulalio et al., 2008). Indeed, we found that miR-223 expression steadily increases during myeloid differentiation (Figure 2B), concomitant with decreasing PRMT4 protein expression. To determine whether miR-223 regulates PRMT4 expression, we transiently transfected $CD34^+$ cells with a short hairpin encoding the mature miR-223 for 24 hr and monitored PRMT4 expression, using siPRMT4 as a short-hairpin-positive control (and a scrambled short hairpin as a negative control). PRMT4 protein levels decreased by 50% in the miR-223-overexpressing cells, similar to that seen in the cells expressing small interfering RNA (siRNA) directed against PRMT4 (Figure 2C).

To validate that PRMT4 is directly targeted by miR-223, we cloned its full-length 3′ UTR into a luciferase reporter plasmid (UTR-WT), using the same construct with a mutated miR-223 targeting sequence (UTR-mut), which cannot bind miR-223, as negative control. We expressed these constructs in 293T cells, with either the miR-223 short hairpin or the control short hairpin and found that miR-223 decreased the luciferase activity of the UTR-WT, with no effect on the UTR-mut reporter plasmid (Figures 2D and S2C). Thus, miR-223 directly targets PRMT4 by binding to its recognition sequence in the 3′ UTR; this suggests that PRMT4 and miR-223 form a regulatory loop to regulate myeloid differentiation.

To determine how important the regulation of PRMT4 expression by miR-223 is for the effects of PRMT4 on myeloid differentiation, we overexpressed the PRMT4-ORF with either the WT or the mutant PRMT4 3′ UTR, in human HSPCs. Although the WT 3′ UTR abrogated the PRMT4 imposed block in myeloid differentiation, when PRMT4 was expressed without the 3′ UTR, or with the mutant 3′ UTR, the block in myeloid differentiation was seen (Figure 2E). These effects correlate with PRMT4 protein levels (Figure 2F) and demonstrate that the regulation of PRMT4 by miR-223 is important to its function during myeloid differentiation.

PRMT4 Represses miR-223 Expression

Given the known transcriptional regulatory role of PRMT4, to determine how PRMT4 controls myeloid differentiation, we first examined the expression level of several differentiation-specific "master" transcription factors, including PU.1, C/EBPα, KLF4, and GATA1 in PRMT4-KD cells. Although we found no significant changes (Figure S3A), we observed a consistent increase in miR-223 expression in the PRMT4-KD cells (Figure 3A). Because upregulation of miR-223 has been reported to promote the myeloid differentiation of leukemia cells (Fazi et al., 2007), we overexpressed miR-223 in normal CB $CD34^+$ cells, and saw a significant increase in CD11b-positive cells (51.2% versus 38.1% for the control cells) (Figure 3B) as well as decreased PRMT4 expression (Figure S2B). We observed decreased miR-223 expression, when PRMT4 is overexpressed (Figure 3C). When we knocked down miR-223 expression, we found a modest but consistent reduction in CD11b-positive cells (38.6% ± 3.1% versus 44.8% ± 1.8%, $p < 0.05$) (Figures 3D and S3B), which suggests that other microRNAs may compensate for miR-223 during myeloid differentiation. This is consistent with the phenotype of miR-223 knockout mice, where miR-223 is important, but not essential, for granulocytic maturation and function (Johnnidis et al., 2008).

To determine whether PRMT4 regulates the transcription of miR-223, we quantified the level of miR-223 primary transcript (pri-miR-223) and found that PRMT4 expression does reciprocally regulate pri-miR-223 levels in $CD34^+$ cells (Figure S3C). In addition, gene expression analysis of PRMT4 KD cells (GSE46056) revealed a gene signature consistent with an upregulation of myeloid differentiation (Figure S3D). Thus, PRMT4 regulates myeloid differentiation, at least in part, by modulating miR-223 expression.

Figure 2. PRMT4 Is a Potential Target Gene of miR-223 during Myeloid Differentiation of HSPCs

(A) PRMT4 protein expression is progressively downregulated during myeloid differentiation (left), whereas PRMT4 mRNA level decreases modestly during myeloid differentiation (right). Isolated $CD34^+$ cells were cultured in myeloid-promoting cytokine containing medium and collected at sequential time points: days (D) 0, 3, 5, and 7. Western blot and qRT-PCR analyses were performed. qRT-PCR data represent the mean ± SD of three independent experiments. $^{**}p < 0.01$ by Student's t test. Tubulin served as the loading control.

(B) miR-223 expression steadily increases during myeloid differentiation. qRT-PCR data represent the mean ± SD of three independent experiments. $^{***}p < 0.001$ by Student's t test.

(C) Overexpression of miR-223 or siRNA directed against PRMT4 lowers PRMT4 protein levels in HSPCs. miR-223, siRNA against PRMT4, and control oligonucleotides were transiently expressed in $CD34^+$ cells and 24 hr postelectroporation; the cells were collected and assayed for PRMT4 expression by western blot analyses.

(D) Putative miR-223 binding site in the PRMT4 3′ UTR is shown at the top (based on TargetScan.org release 5.2). Luciferase activity in 293T cells cotransfected with a reporter plasmid containing either the wild-type 3′ UTR-PRMT4 or the mutated 3′ UTR (3-UTR-mut, which lacks the seed miR-223 sequence) with or without miR-223. Renilla luciferase values are normalized based on the value of firefly luciferase. Mean ± SD from three independent experiments is shown. $^{***}p < 0.001$ by Student's t test.

(E) Control of PRMT4 expression by miR-223 is essential to regulate PRMT4 function during normal myeloid differentiation. Human CB $CD34^+$ cells were transduced with retroviruses expressing control-GFP alone; GFP-PRMT4-ORF or GFP-PRMT4 3′ UTR or GFP-PRMT4 3′ UTR-mut. Sorted GFP^+ $CD34^+$ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD11b expression. The percentage of CD11b-positive cells was quantified as mean ± SD based on three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$ by Student's t test.

(F) qRT-PCR and western blot analyses of PRMT4 expression in control $CD34^+$ cells or $CD34^+$ cells expressing PRMT4-ORF, PRMT4 3′ UTR, or PRMT4 3′ UTR-mut. Tubulin served as the loading control.

See also Figure S1.

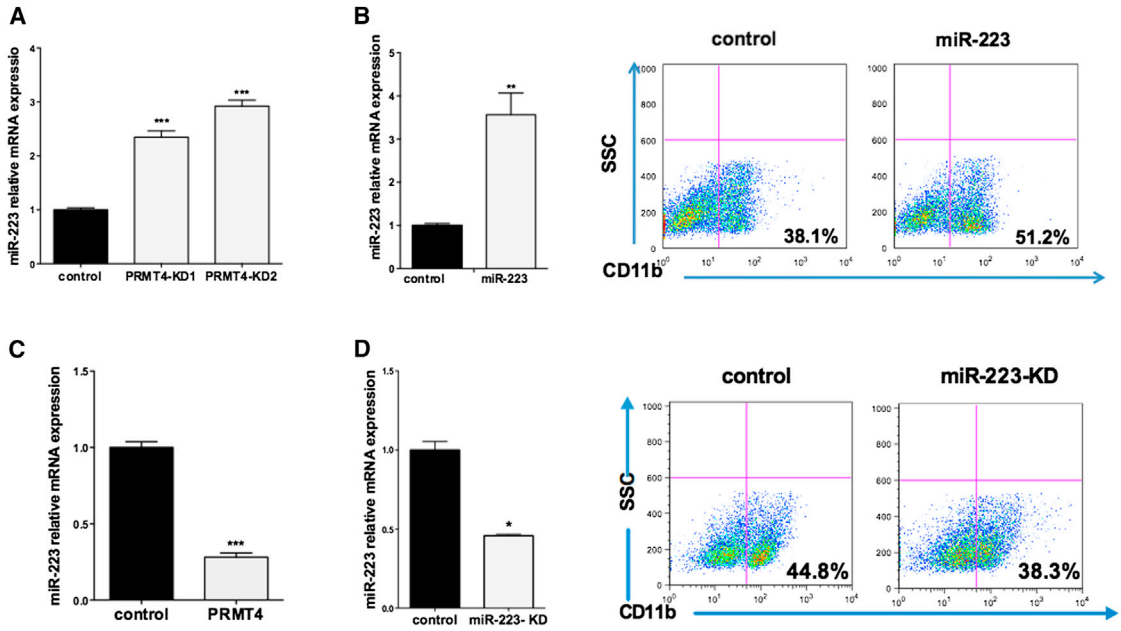


Figure 3. PRMT4 Regulates miR-223 Expression

(A) PRMT4 downregulation leads to upregulation of miR-223 expression. qRT-PCR data represent the mean ± SD of three independent experiments. ***$p < 0.001$ by Student's t test.

(B) Overexpression of miR-223 in HSPCs enhances myeloid differentiation. (left) qRT-PCR data represent the mean ± SD of three independent experiments. **$p < 0.01$ by Student's t test. (right) Cells were plated in myeloid differentiation promoting culture and assayed for CD11b expression after 7 days.

(C) Overexpression of PRMT4 diminishes miR-223 expression. qRT-PCR data represent the mean ± SD of three independent experiments. ***$p < 0.001$ by Student's t test.

(D) Knockdown of miR-223 in HSPCS slightly impairs myeloid differentiation. (left) qRT-PCR data represent the mean ± SD of three independent experiments. *$p < 0.05$ by Student's t test. (right) Cells were plated in myeloid differentiation promoting culture and assayed for CD11b expression after 7 days.

See also Figure S3.

RUNX1 Is Methylated by PRMT4 on R223

PRMT4 is generally thought to act as a transcriptional co-activator; however, we found that PRMT4 functions as a repressor of miR-223 expression in hematopoietic cells. To determine how this ''coactivator'' suppresses gene expression, we hypothesized that PRMT4 modulates the activity of a regulatory transcription factor. Based on the report that the leukemogenic AML1-ETO fusion protein represses miR-223 expression via a RUNX1 consensus-binding site (CBS) in a specific regulatory region (Fazi et al., 2007), we examined the miR-223 promoter and found another RUNX1 CBS close to the miR-223 promoter (Fukao et al., 2007). Given the presence of these CBS, we investigated whether wild-type RUNX1 also regulates miR-223 transcription. We knocked down RUNX1 in $CD34^+$ cells using two different shRNAs and observed upregulation of pri-miR-223 and pre-miR-223 expression (Figure S4A), indicating that RUNX1 can transcriptionally inhibit miR-223 expression in HSPCs. Given that both RUNX1 and PRMT4 repress miR-223 transcription, we examined whether RUNX1 is arginine methylated by PRMT4. Using an in vitro methylation assay, we identified one specific site in RUNX1, R223, which is methylated by PRMT4. This arginine residue is located just C-terminal to the RUNX1 DNA binding domain (Figure S5A) and is conserved among vertebrates (Figure S5B).

To determine whether RUNX1 is methylated at R223 by PRMT4 in vivo, we generated a methylation-specific methyl-R223RUNX1 antibody, which recognizes an asymmetric dimethylated R223RUNX1 peptide, but not the unmethylated peptide (data not shown). We confirmed the specificity of the antibody by overexpressing Flag-RUNX1 or the Flag-RUNX1R223K mutant protein in 293T cells with or without HA-PRMT4, or with an enzymatically dead form of PRMT4 (PRMT4EQ). Methylation of RUNX1 at R223 was strongly detected when both RUNX1 and PRMT4 were overexpressed (Figure 4A, lane 1) or when RUNX1 was overexpressed by itself (lane 5). No methylation was detected when either the mutant R223K-RUNX1 protein was expressed or when PRMT4EQ was overexpressed (Figure 4A, lanes 2–4). Thus, we conclude that PRMT4 methylates RUNX1 at residue R223 in vivo. The same experiment showed the physical interaction between RUNX1 and PRMT4, using an HA antibody to detect PRMT4 in the RUNX1-containing immunoprecipitate; we detected an interaction between RUNX1 and PRMT4 when RUNX1 and PRMT4EQ were coexpressed or when the R223K-RUNX1 mutant was overexpressed with PRMT4 (or PRMT4EQ) (Figure 4A, IP lanes 2–4). Minimal interaction was seen when RUNX1 overexpressed with WT-PRMT4 (Figure 4A, IP lane 1), suggesting that PRMT4 preferably associates with the non-methylated form of RUNX1.

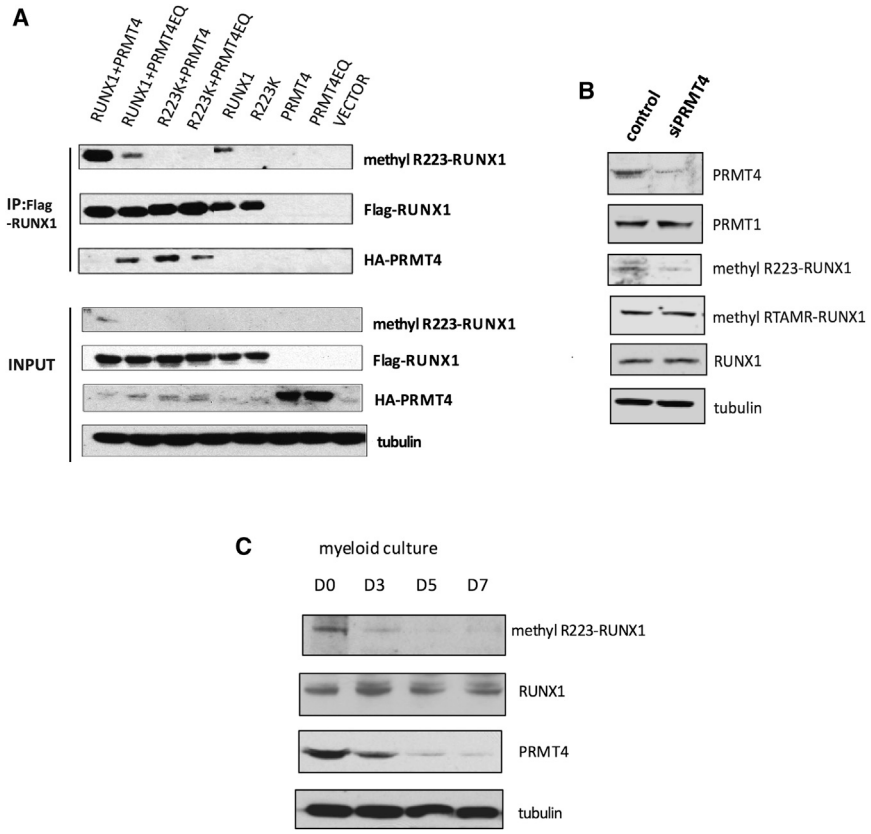


Figure 4. RUNX1 Is Arginine Methylated by PRMT4 on R223

(A) In vivo methylation of RUNX1 by PRMT4 was detected using a methyl-RUNX1-specific antibody. The full-length Flag-RUNX1 or Flag-RUNX1-R223K mutant cDNAs were overexpressed in 293T cells, with or without HA-tagged-WT-PRMT4, or an enzymaticallly dead PRMT4 mutant (PRMT4EQ). Immunoprecipitation was performed using a Flag antibody and immunoblotting with FLAG or HA antibodies. Wild-type RUNX1 (lanes 1 and 2), but not the R223K mutant protein (lanes 3 and 4) is methylated by wild-type PRMT4. The physical interaction between RUNX1 and PRMT4 is detected when RUNX1 is overexpressed with PRMT4EQ, but not WT-PRMT4, and when R223K is overexpressed with PRMT4 or PRMT4EQ (lanes 1–4 in the third row). Tubulin served as the loading control.

(B) Knockdown of PRMT4 in HEL cells using siRNA reduces the level of endogenous methylR223-RUNX1, without altering total RUNX1 levels or the methylation of RUNX1 at the RTAMR motif.

(C) The level of RUNX1R223 methylation decreases during myeloid differentiation, without changes in total RUNX1 expression but concordant with changes in PRMT4 protein levels.

See also Figure S4.

To determine whether PRMT4 is the major enzyme catalyzing RUNX1R223 methylation in vivo, we knocked down PRMT4 using siRNA in HEL cells; we observed a marked reduction in RUNX1 R223 methylation but no change in RUNX1 methylation at the RTAMR site, which we previously showed is methylated by PRMT1 (Figure 4B). Thus, PRMT4 appears to be the major methyltransferase that asymmetrically methylates RUNX1 at R223 in vivo.

Given the downregulation of PRMT4 protein levels during myeloid differentiation, we examined whether RUNX1R223 methylation is similarly downregulated, and indeed, as human $CD34^+$ cells differentiate and PRMT4 expression diminishes, the level of methylR223-RUNX1 decreases, even though total RUNX1 protein levels are unchanged (Figure 4C). To determine whether R223 methylation is important for RUNX1 transcriptional activity, we compared the transcriptional regulatory activity of RUNX1 R223K versus WT-RUNX1 on the pri-mir-223 expression in CB $CD34^+$ cells. Although RUNX1 overexpression repressed pri-miR-223 transcript levels, the RUNX1 R223K mutant did not (Figure S4B). Thus, the repression effects of PRMT4 on miR-223 expression appear to relate to its ability to posttranscriptionally modify RUNX1 on R223.

Methylation of RUNX1 at R223 Regulates Its Interaction with DPF2

We hypothesized that methylation of R223 in RUNX1 by PRMT4 affects its protein-protein interactions, and performed peptide pull-downs using both a methyl-R223 RUNX1 peptide and an unmodified RUNX1 peptide as bait, followed by mass spectrometry, to identify proteins that interact with unmethylated or R223 methylated RUNX1 protein. We identified several proteins that interact with the R223 methyl peptide (Table S1), including DPF2 (double PhD Finger 2), a widely expressed member of the d4 protein family that is characterized by the presence of a tandem plant homodomain (PHD domain). DPF2 was recently reported to interact with lysine-acetylated histones and acts as a corepressor (Matsuyama et al., 2010). We first verified the interaction of DPF2 with the methyl-R223 RUNX1 peptide in a peptide pull-down assay: DPF2 preferentially bound to the arginine-methylated peptide, whereas PRMT4 preferred the nonmethylated RUNX1 peptide, similar to the interaction of PRMT4 with full-length RUNX1 (Figure 5A).

To determine whether RUNX1 and DPF2 associate in vivo, we performed coIP assays using two different RUNX1 antibodies and consistently immunoprecipitated the endogenous DPF2 protein, confirming their in vivo interaction (Figure 5B). To show that this interaction depends on arginine methylation, we treated HEL cells with adenosine-2′,3′-dialdehyde (AdOx) (20 μM), a panmethyltransferase inhibitor, which significantly reduced RUNX1 methylation after 16 hr (Figure 5D). This treatment abrogated the interaction of RUNX1 with DPF2 (Figure 5D, compare lane 2 and 4). To further demonstrate that DPF2 interacts specifically with R223-methylated RUNX1, we overexpressed Flag-RUNX1 and Flag-RUNX1R223K and performed coimmunoprecipitation studies using anti-Flag beads. DPF2 associated with Flag-RUNX1, but not Flag-RUNX1R223K (Figure 5C, compare

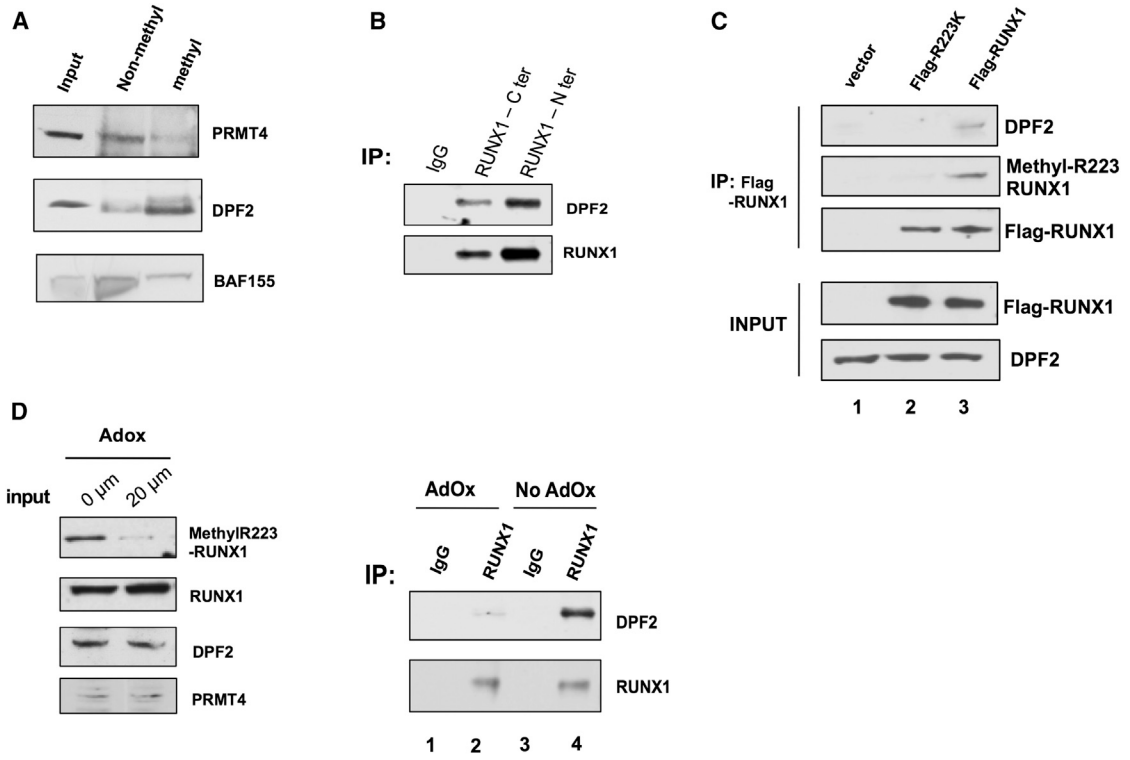


Figure 5. Methylation of RUNX1 at R223 Regulates Its Interaction with DPF2

(A) DPF2 is preferably pulled down by a methylR223-RUNX1 peptide, whereas the unmodified R223-RUNX1 peptide interacts more strongly with PRMT4. BAF155 is used as a control, which shows no preference for binding to either the modified or the unmodified peptide. Input: 10% of the used nuclear extract.

(B) The endogenous RUNX1 and DPF2 proteins physically interact in vivo. Two RUNX1 antibodies were used to immunoprecipitate DPF2 from HEL cell nuclear extract. DPF2 was detected using a DPF2 antibody. Preimmune rabbit serum was used as control.

(C) The interaction between RUNX1 and DPF2 is dependent on the RUNX1 methylation status. Overexpressed Flag-RUNX1 is immunoprecipitated using a Flag antibody. DPF2 is coprecipitated with Flag-RUNX1, but not the Flag-R223K mutant (compare lane 1 versus lane 3).

(D) Treatment of cells with AdOx reduces the level of RUNX1 methylation (input), abrogating its interaction with DPF2 (compare lane 2 versus lane 4).

See also Figure S5.

lanes 2 and 3), again demonstrating that the interaction of DPF2 with RUNX1 is dependent on the R223 methylation.

miR-223 Expression Is Regulated by a RUNX1-Methylation-Dependent Repressor Complex

Given the decrease in RUNX1R223 methylation during myeloid differentiation and the demonstrated interaction of R223-methylated RUNX1 with DPF2, we hypothesized that the DPF2-RUNX1 interaction regulates miR-223 expression. We examined the binding of RUNX1, PRMT4, and DPF2 to miR-223 regulatory regions by chromatin immunoprecipitation (ChIP) assays using HSPCs and in vitro differentiated myeloid cells, which correspond to the states where miR-223 is low and RUNX1 R223 methylation high (i.e., the HSPC stage), and where miR-223 is high, and RUNX1 R223 methylation low (i.e., in differentiated myeloid cells) (Figures 6A and 6B). Using primer pairs that cover much of the miR-223 putative regulatory regions (as depicted in Figure 6C and Table S2), we detected RUNX1, methyl-R223 RUNX1, and DPF2 at the pre-miR-223 promoter region, when the cells were at the "stem/progenitor" stage and miR-223 was minimally expressed (Figure 6D). However, in the differentiated cells where miR-223 was actively transcribed, we found RUNX1 and not methyl-R223 RUNX1 (Figure 6E). PRMT4 is found throughout the miR-223 regulatory region in the HSPCs but not in the differentiated cells, with a slight peak at region 4. DPF2 protein is clearly expressed in the differentiated cells (Figure 6A), but it is not found at the miR-223 regulatory regions (Figure 6E), suggesting that its recruitment to the miR-223 promoter depends on the methylation status of RUNX1. Thus, recruitment of DPF2 by methyl-R223 RUNX1 may dictate the transcriptional effects of RUNX1 on the miR-223 locus.

DPF2 Inhibits miR-223 Expression and Myeloid Differentiation

We next examined whether DPF2 can directly regulate miR-223 expression. We achieved a 50% knockdown of DPF2 mRNA and protein using shRNA (Figure 6F) and found a 3-fold increase in miR-223 expression (Figure 6G) and a decrease in PRMT4 protein levels. The DPF2 KD cells also showed enhanced myeloid differentiation (based on CD11b expression, Figure 6H) and decreased clonogenic potential (Figure S6) similar to what

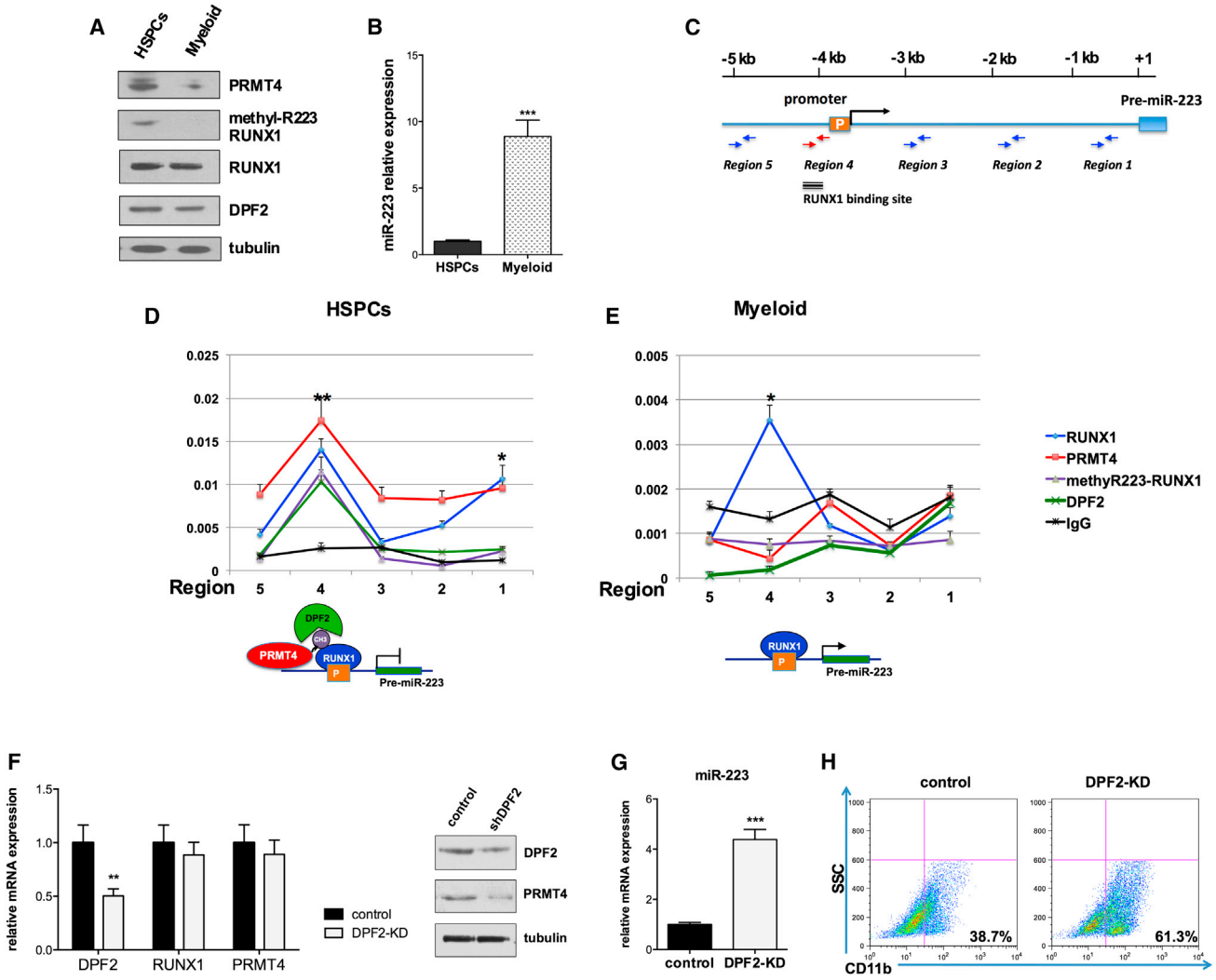


Figure 6. The RUNX1 Methylation-Dependent Repressor Complex Regulates miR-223 Expression

(A) Western blot analysis quantified the levels of expression of PRMT4, methyl R223-RUNX1, RUNX1, and DPF2 in CD34$^+$ cells maintained in basic culture or in cells cultured in myeloid differentiation-promoting medium for 7 days. Tubulin served as the loading control.

(B) The level of miR-223 expression in the HSPCs and myeloid differentiated cells. qRT-PCR data represent mean ± SD of three independent experiments. ***$p < 0.001$ by Student's t test.

(C) A schematic diagram of the miR-223 promoter. The location of the RUNX1 consensus binding sites and the primers used for the ChIP assays are shown.

(D and E) ChIP assays show the presence of a methyl-R223 RUNX1-dependent complex at the promoter of miR-223 in HSPCs (D), but not in myeloid differentiated cells (E). Upper panel: enrichment of proteins of interest at miR-223 regulatory regions was assayed by qRT-PCR and shown as percentage of the genomic input DNA. *$p < 0.5$; **$p < 0.01$ by Student's t test. Lower panel: diagrams demonstrating the recruitment of RUNX1, methylR223-RUNX1, and DPF2 to the miR-223 promoter.

(F) Efficient knockdown of DPF2. CD34$^+$ cells were transduced with lentiviruses expressing a control (scrambled) shRNA or shRNA directed against DPF2. GFP-positive cells were sorted 3 days after transfection and collected to perform qRT-PCR and western blot analyses. qRT-PCR data represent the mean ± SD of three independent experiments. **$p < 0.01$ by Student's t test.

(G) Downregulation of DPF2 increases miR-223 expression. qRT-PCR analysis of miR-223 in control and DPF2 knockdown CD34$^+$ cells. The data represent the mean ± SD of three independent experiments. ***$p < 0.001$ by Student's t test.

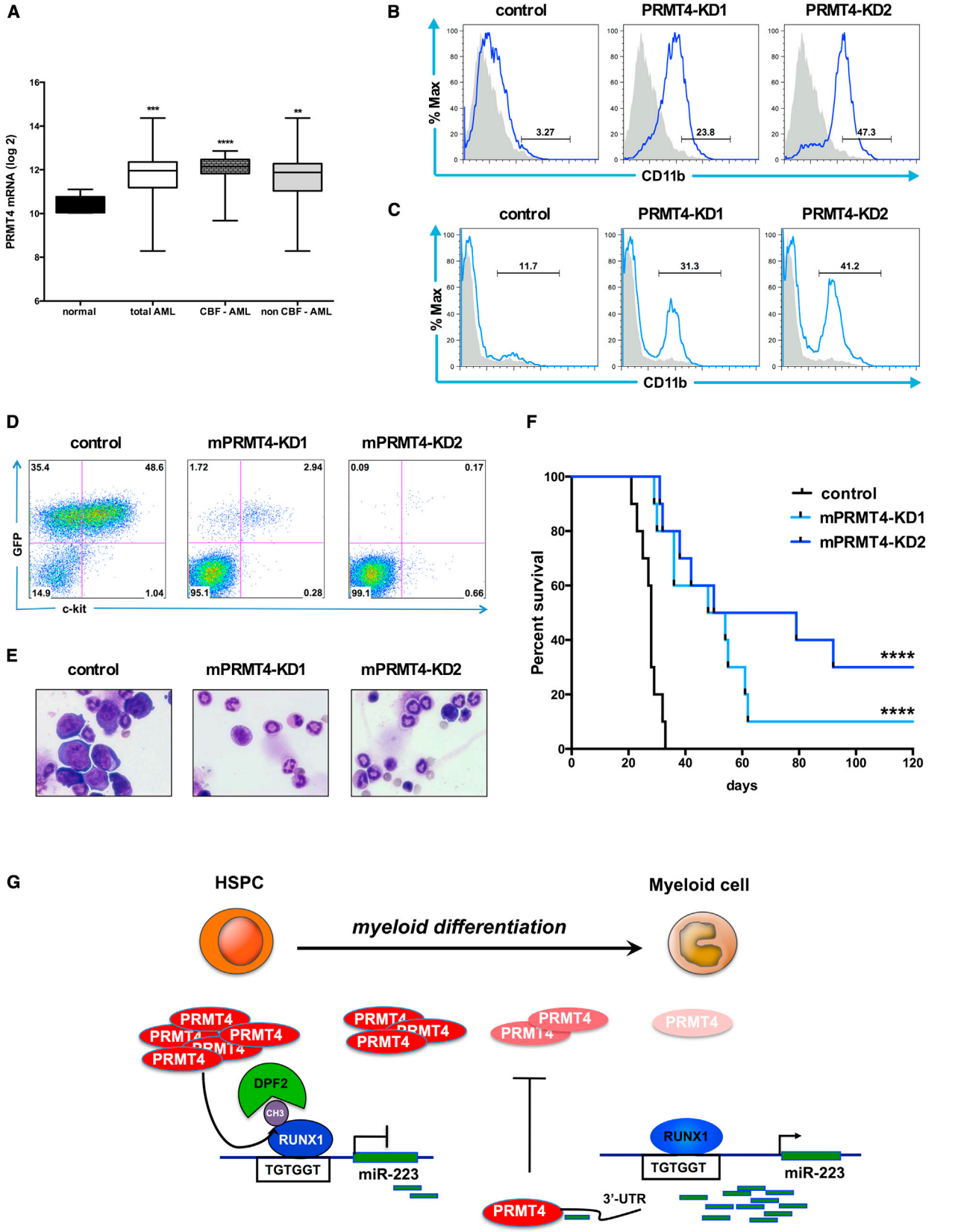
(H) Downregulation of DPF2 accelerates the myeloid differentiation of HSPCs. GFP$^+$ CD34$^+$ cells were cultured in myeloid differentiation promoting medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD11b expression.

See also Figure S6.

occurred when we knocked down PRMT4. This places PRMT4 and DPF2 in a RUNX1 containing complex that downregulates miR-223 expression and impairs the myeloid differentiation of HSPCs.

Knockdown of PRMT4 Is Sufficient to Induce Myeloid Differentiation in AML Cell Lines

As PRMT4 expression impairs the differentiation of human HSPCs, we examined the level of PRMT4 expression in a cohort



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of 318 AML patient samples (GSE24505) (Figueroa et al., 2010). PRMT4 levels were significantly upregulated in the AML samples, compared to the control group (n = 5) (Figure 7A). A high level of PRMT4 expression was seen in core-binding factor (CBF) AMLs (which express either AML1-ETO or CBFβ-SMMHC), which exhibit low-level miR-223 expression, but, overall, about 70% of the AML patients had at least a 2-fold increase in PRMT4 expression (Figure S7A).

Several studies have shown that overexpression of miR-223 can promote the granulocytic differentiation of NB4 acute promyelocytic leukemia (APL) cells (Fazi et al., 2005). We knocked down PRMT4 in NB4 cells and triggered myeloid differentiation with a significant increase in the number of CD11b-positive cells (3.3% control versus 23.8% and 47.3%) (Figure 7B) as well as changes in cellular morphology and an upregulation of miR-223 expression (Figures S7B and S7C). A similar induction of differentiation was also observed in the ATRA-resistant NB4-R4 cells following PRMT4-KD (Rosenauer et al., 1996) (Figures S7D and S7E). Because therapeutic targeting of most leukemia fusion proteins, including AML1-ETO remains elusive, we tested whether targeting PRMT4 can promote the differentiation of AML1-ETO-expressing cells (Figure 7A). We knocked down PRMT4 in t(8;21)-positive Kasumi-1 cells and found increased differentiation (Figures 7C and S7E). We also saw significant apoptosis of all three cell lines when PRMT4 is knocked down (Figure S7F), suggesting that PRMT4 not only impairs the differentiation of these leukemia cells, but it is also critical for their survival.

Knockdown of PRMT4 Reduces the Leukemia Burden In Vivo

To investigate the in vivo role of PRMT4 in leukemogenesis, we used shRNA-expressing lentiviruses to knock down PRMT4 in the AML1-ETO9a (AE9a)-driven mouse AML model (Yan et al., 2006; Wang et al., 2011). Leukemia cells growing in culture were transduced with two different shRNAs, and ≥70%–80% KD was achieved (Figure S7G). The transduced AE9a-mPRMT4KD and control cells were injected into sublethally irradiated C57Bl/6 mice (day 0). We observed decreased numbers of immature GFP^+ $c\text{-}kit^+$ blast cells in the peripheral blood of the AE9a-mPRMT4KD mice compared to the control mice at week 3 (Figure 7D). Morphological analysis of the bone marrow (Figure 7E) and peripheral blood (Figure S7I) also showed a marked reduction in blast cells at week 4 with lower white blood cell counts, and less anemia and thrombocytopenia (Figure S7H), compared to the AE9a-control mice. This translated to a significant increase in median survival, from 28 days for the control group, to 51 days and 64.5 days for the AE9a-mPRMT4-KD1 and AE9a-mPRMT4-KD2 groups, respectively ($p < 0.0001$; Figure 7F). This demonstrates an important role for PRMT4 in leukemogenesis and identifies it as an important therapeutic target.

DISCUSSION

We have found that PRMT4 is highly expressed in HSPCs, where it functions as an inhibitor of myeloid differentiation (Figure 7G). In these cells, PRMT4 methylates RUNX1 at R223, promoting the assembly of a DPF2-containing transcriptional corepressive complex and repressing transcription at the miR-223 locus. As HSPCs undergo myeloid differentiation, PRMT4 expression decreases, reducing the amount of R223-methyl RUNX1, which, in turn, decreases the presence of DPF2 at the miR-223 promoter region, thereby allowing miR-223 to be transcribed. The ability of miR-223 to target the 3′ UTR of PRMT4 allows the further upregulation of miR-223 expression, which further decreases PRMT4 and sustains the myeloid differentiation process. Although PRMT4 promotes differentiation in several biological systems including T cell, adipocyte, and muscle development, it blocks differentiation in the hematopoietic system,

Figure 7. Downregulation of PRMT4 Is Sufficient to Induce Myeloid Differentiation in Leukemia Cells

(A) PRMT4 expression is upregulated in AML patient samples. The graph shows the log2 expression of PRMT4 from transcript profiling of $CD34^+$ bone marrow cells isolated from five healthy donors (normal) or 318 AML patients. CBF, core-binding factor. CBF-AML n = 57. Non-CBF-AML n = 261. ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ by Student's t test.

(B) Knockdown of PRMT4 triggers the myeloid differentiation of NB4 cells. NB4 cells were transduced with lentiviruses expressing control (scrambled) shRNA or shRNA directed against PRMT4. Sorted GFP-positive cells were cultured for 3 days prior to FACS analysis of CD11b expression. Quantitative data are shown in Figure S7D.

(C) Knockdown of PRMT4 triggers the myeloid differentiation of Kasumi-1 cells. Kasumi-1 cells were transduced with lentiviruses expressing control (scrambled) shRNA or shRNA directed against PRMT4. Sorted GFP-positive cells were cultured for 3 days prior to FACS analysis of CD11b expression. Quantitative data are shown in Figure S7D.

(D) FACS analysis showed far fewer GFP^+ $ckit^+$ cells in peripheral blood (PB) of the mice transplanted with AE9a-mPRMT4-KD cells in compare to AE9a-control cells at week 3. AE9a expressing mouse leukemia cells were transduced with shRNA scramble control (AE9a-control), or shRNAs against PRMT4 (AE9a-mPRMT4-KD1 and mPRMT4-KD2). Transduced cells were sorted for expression of RFP and the sorted cells were injected into recipient mice that had received sublethal irradiation.

(E) Bone marrow (BM) morphology shows marked reduction in the number of leukemic cells in mice transplanted with AE9a-mPRMT4-KD cells, compared to AE9a-control cells.

(F) Knockdown of PRMT4 prolongs the survival of AE9a transplanted mice. The median survival was extended in the knockdown groups, compared to the control group (54 days and 64.5 days versus 28 days $p < 0.0001$).

(G) A schematic model showing how PRMT4 regulates myeloid differentiation of human HSPCs. PRMT4 and miR-223 form a regulatory loop that is critical for myeloid differentiation. PRMT4 inhibits myeloid differentiation by assembling a methyl R223-RUNX1-DPF2 repressor complex that suppresses miR-223 expression. When HSPCs undergo myeloid differentiation, PRMT4 expression is downregulated, releasing the miR-223 locus from transcription repression, allowing it to be transcribed. At that stage, the higher expression of miR-223 targets the PRMT4 3′ UTR to further decrease PRMT4 expression, thereby reinforcing the myeloid differentiation process.

See also Figure S7.

allowing HSPCs to maintain stemness. This role is consistent with its role in embryonic stem cells, where PRMT4 functions to maintain pluripotency (Torres-Padilla et al., 2007; Wu et al., 2009).

The changes in PRMT4 expression and miR-223 during myeloid differentiation allowed us to define a link between an arginine methyltransferase and the expression of a microRNA (miR-223), with PRMT4 and miR-223 forming a regulatory loop to influence myeloid differentiation. MicroRNAs that target enzymes involved in epigenetic regulation are being identified, and PRMT4 can join the list of targets, which thus far includes Dnmt3A (miR-29) (Fabbri et al., 2007; Garzon et al., 2009) and EZH2 (miR-101) (Varambally et al., 2008). Although we have identified the specific sequence in the PRMT4 3′ UTR that contributes to its regulation by miR-223, the 3′ UTR region of PRMT4 has binding sequences for other microRNAs that could also target its expression. Whether these microRNAs also contribute to the regulation of PRMT4 during hematopoiesis will require further study.

The initial signals that trigger the downregulation of PRMT4 expression, which helps drive the process of myeloid differentiation, remain to be determined. Once the decrease in PRMT4 activity occurs, increased expression of miR-223 (and likely other targets of the methyl-RUNX1 repressor complex) occurs, which can further downregulate PRMT4 protein levels and activity, and promote differentiation. This regulatory feedback loop therefore pushes the differentiation process forward. It is known that PRMT4 enzymatic activity can be regulated by posttranslational modifications (Cheung et al., 2008; Higashimoto et al., 2007); thus, upstream signaling pathways (such as PI3K/AKT; Supplemental Results) could control PRMT4 enzymatic activity and expression.

A fundamental aspect of transcriptional regulation has been to define how a given protein can function either as an activator or a repressor. We have recently shown that AML1 (RUNX1)-ETO, a well-known leukemia-associated TF fusion protein generally thought to function as a transcriptional repressor, has activator functions as well that are critical to its leukemogenic properties (Wang et al., 2011). Our study of PRMT4 provides further evidence for a flexible model of how proteins regulate gene expression. PRMT4 has been thought of as a "secondary" coactivator molecule that helps activate transcription of its target genes via methylation of histone H3. We have identified a transcriptional repressor function for PRMT4 and provide a molecular basis for this function, which involves the methylation of a nonhistone substrate, namely RUNX1. The interaction of PRMT4 with RUNX1 appears to be transient, i.e., a kind of "hit and run." However, the recruitment of PRMT4 to the chromatin of its target genes could be more stable, either due to its binding histones or other chromatin associated factors.

It is clear that RUNX1 can assemble a variety of multiprotein complexes that affects its transcriptional regulatory functions. These complexes are regulated by various posttranscriptional modifications. The association of RUNX1 with mSIN3A is disrupted by the PRMT1-dependent methylation of RUNX1 on R206 and R210 (Zhao et al., 2008). Similarly, the methylation of C/EBPβ by PRMT4 interfered with its association with both the SWI/SNF and Mediator complexes (Kowenz-Leutz et al., 2010). In contrast to that model, we show that the methylation of RUNX1 by PRMT4 actually promotes protein-protein interactions.

We found the preferential binding of DPF2 to R223-methylated RUNX1 and that, by recruiting DPF2, RUNX1 can repress miR-223 expression. This function of DPF2 is consistent with its ability to act as a cosuppressor of nuclear receptor-mediated transcription regulation, by binding HDAC1 (Matsuyama et al., 2010). Although DPF2 has been implicated in transcriptional regulation, little is known about its biological functions. Here, DPF2 appears to be another important regulator of myeloid differentiation that can cooperate with PRMT4 to maintain the "stemness" of HSPCs. As both PRMT4 (Torres-Padilla et al., 2007; Wu et al., 2009) and DPF2 are expressed in ES cells (Ho et al., 2009), they may also cooperatively regulate gene expression in ES cells.

Targeting of the differentiation (and apoptotic) processes has become a promising therapeutic approach in the treatment of hematologic malignancies like AML, which are characterized by a block in differentiation. We were able to differentiate myeloid leukemic cells by knocking down PRMT4 and observed this effect even in ATRA-resistant cell lines. By utilizing the AML1-ETO driven leukemia model, we showed that knocking down of PRMT4 not only induced myeloid differentiation but also triggered apoptosis, leading to improved survival in an in vivo mouse AML model. These findings strongly suggest that targeting PRMT4 function could hold potential as a novel therapy of acute myelogenous leukemia.

EXPERIMENTAL PROCEDURES

Purification, Infection, and Culture of HSPC-CD34$^+$ Cells

CD34$^+$ HSPCs were purified by positive selection using the Midi MACS (magnetic-activated cell sorting) LS$^+$ separation columns and isolation Kit (Miltenyi) starting with mononuclear cells that were isolated from cord blood (CB) by Ficoll-Hypaque Plus density centrifugation. CD34$^+$ cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Cellgro) 20% BIT 9500 medium (STEMCELL Technologies) supplemented with SCF (100 ng/ml), FLT-3 ligand (10 ng/ml), IL-6 (20 ng/ml), and TPO (100 ng/ml) as the basic culture. CD34$^+$ cells were infected with high-titer lentiviral concentrated suspensions, with 8 μg/ml polybrene (Sigma-Aldrich). To differentiate HSPCs, cells were cultured under the myeloid-promoting conditions: SCF (100 ng/ml), FLT-3 ligands (10 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), GM-CSF (20 ng/ml), and G-CSF (20 ng/ml) and the erythroid-promoting conditions Epo (6 IU/ml) and SCF (100 ng/ml). Cytokines were purchased from PeproTech.

Peptide Pull-Down Assay

Methylated (Acetyl-TPNPR [Asymmetric-dimethyl] ASLNHS-C-amide) and nonmethylated (Acetyl-TPNPRASLNHS-C-amide) peptides were synthesized, quantified, and conjugated to SulfoLink agarose (Pierce). For each pull-down reaction, 10 mg of HEL cell nuclear extract was used with 10 μg peptide bound beads in H lysis buffer (20 mM HEPES [pH 7.9], 150 mM NaCl, 1 mM $MgCl_2$, 1% NP40, 10 mM NaF, 0.2 mM $NaVO_4$, 10 mM β-glycerol phosphate, 5% glycerol) with freshly added 1 mM DTT and proteinase inhibitor cocktail (Roche). After rotating overnight at 4°C, the beads were washed five times with the binding solution. The bound protein was then eluted with 1 × SDS sample buffer and analyzed on 4%–12% NUPAGE gels.

ChIP Assays

Approximately 4 × 10^6 cells were used per ChIP reaction after crosslinking with 1% formaldehyde for 10 min at room temperature. ChIP assays were performed according the previously reported methodology (Zhao et al., 2008).

After purification, the associated DNA was subjected to quantitative RT-PCR (qRT-PCR) to detect specific DNA sequences. Quantitative results are represented as percentages relative to 5% DNA input. Table S2 provides primer sequences.

In Vivo Transplantation of AE9a Leukemia Cells

AE9a-expressing mouse leukemia cells were generated based on the work of Wang et al. (2011). These cells were transduced with lentiviruses expressing RFP and shRNAs against PRMT4 or a scrambled control shRNA. Transduced cells were sorted for RFP positivity, and 10^5-sorted cells were injected into female C57Bl/6 recipient mice that has been sublethally irradiated with 475 cGy via tail vein. All animal studies were performed on IACUC approved animal protocols.

Statistic

Statistical analyses were carried out using Prism 5.0 for Macintosh. All data are shown as mean ± SD. The mean values of each group were compared by Student's t test.

ACCESSION NUMBERS

RNA sequencing data has been deposited in the NCBI Gene Expression Omnibus under accession number GSE46056.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, Supplemental Results, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.11.025.

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Gene Section
Review

ELF4 (E74-like factor 4 (ets domain transcription factor))

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Abstract

Review on ELF4, with data on DNA/RNA, on the protein encoded and where the gene is implicated.

Identity

Other names: ELFR, MEF

HGNC (Hugo): ELF4

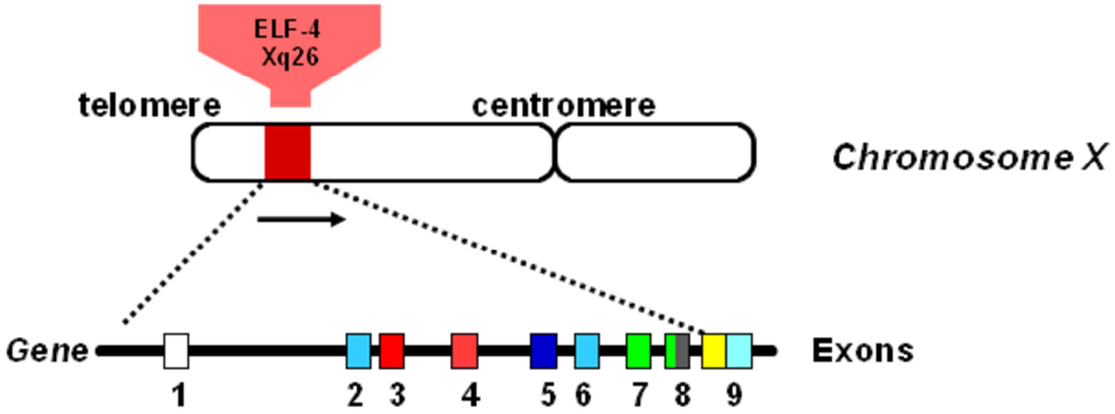
Location: Xq26.1

Local order: The ELF4 gene maps on chomosome X at position +1805468958720.00 (interpolated), at Xq26 according to Entrez Gene. In AceView, it covers 46,69 kb, from 129244687 to 129197994, on the reverse strand.

DNA/RNA

Description

The gene encompasses 45 kb of DNA; 9 exons.
The ELF4 gene contains 12 distinct gt-ag introns. Transcription produces 5 alternatively spliced mRNAs.
There are 2 probable alternative promoters, 2 non overlapping alternative last exons and 2 validated alternative polyadenylation sites.
The mRNAs appear to differ by truncation of the 3' end, presence or absence of 3 cassette exons, overlapping exons with different boundaries, splicing versus retention of 2 introns.



The ELF4 gene is located on the X-chromosome (Xq26) and is transcribed from telomere to centromere. The ELF4 gene contains 8 coding exons and 1 non-coding exon.

The ELF4 protein contains the following domains: (A) transactivation domain, (B) AML1 interacting domain, (C) ETS domain, (D) serine/threonine rich region and (E) proline rich region.

Transcription

4190 bp mRNA; 1991 bp open reading frame.

Pseudogene

No observed pseudogenes.

Protein

Description

663 amino acids; 70730 Da protein; numerous posttranslational modifications: phosphorylation, sumoylation and ubiquitination (Miyazaki et al., 2001; Liu et al., 2006; Suico et al., 2006). ELF4 contains, from N-term to C-term, transactivation domain (53-86), AML1 interacting domain (87-206), ETS domain (207-291), serine/threonine rich region (306-347) and proline rich region (477-517) (Miyazaki et al., 1996; Mao et al., 1999; Suico et al., 2002; Lacorazza and Nimer, 2003).

Expression

Abundantly expressed in the placenta and in a variety of myeloid leukemia cell lines. Moderate levels of expression in heart, lung, spleen, thymus, peripheral blood lymphocytes, ovary and colon. Lower levels of expression in Jurkat T-cells and other T-cell lines and no expression in brain (Lacorazza and Nimer, 2003).

Localisation

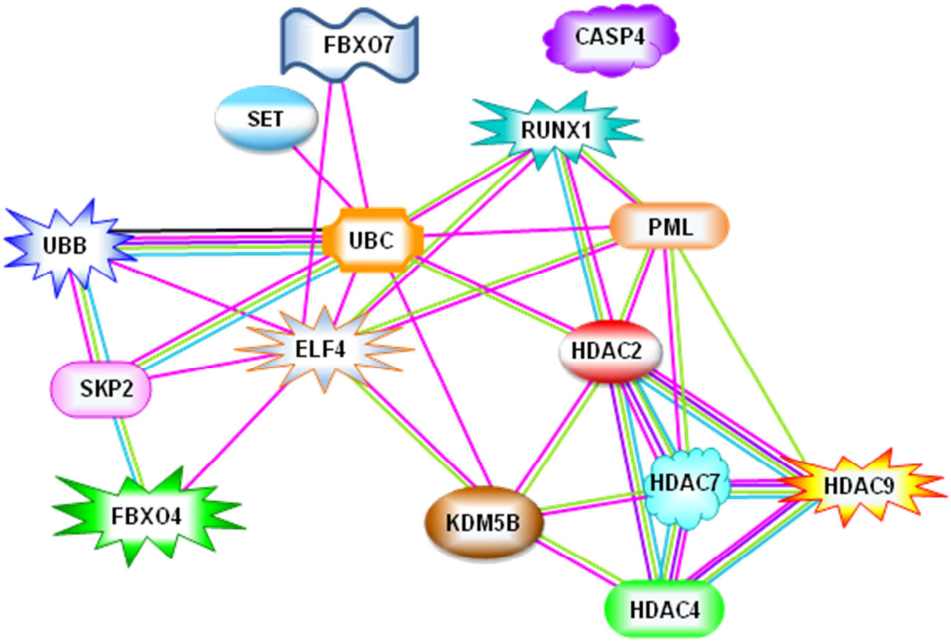
Nucleus, PML body. Accumulation into PML nuclear bodies is mediated by PML (Suico et al., 2004a; Suico et al., 2004b).

Function

ELF4 is a member of the ETS family of transcription factors (TF) with transcription activating properties (Lacorazza and Nimer, 2003). ELF4 binds to DNA sequences containing the consensus 5'-WGGA-3' and transactivates promoters of the hematopoietic growth factor genes CSF2, IL3, IL8, and of the bovine lysozyme gene (Miyazaki et al., 1996; Mao et al., 1999; Hedvat et al., 2004; Suico et al., 2004). ELF4 acts synergistically with RUNX1 to transactivate the IL3 promoter (Mao et al., 1999).

It also transactivates the PRF1 promoter in natural killer (NK) cells (Lacorazza et al., 2002).

ELF4 has important molecular functions, including protein binding, transcription activator activity, sequence-specific DNA binding, transcription factor activity. ELF4 interacts with multiple proteins, including Cyclin A/CDK2 kinase complex, FBXO4, FBXO7, PML, RUNX1, SKP2 and UBB (Miyazaki et al., 1996; Mao et al., 1999; Miyazaki et al., 2001; Liu et al., 2006; Suico et al., 2006).



ELF4 interacts with multiple proteins and plays important role in development and oncogenesis.

ELF4 has been implicated in widely divergent physiological and pathological processes (such as development and oncogenesis).
ELF4 plays an important role in the development and function of NK and NK T-cells and in innate immunity (Lacorazza et al., 2002).
It controls the proliferation and homing of CD8+ T-cells via the Kruppel-like factors KLF4 and KLF2 (Yamada et al., 2009; Yamada et al., 2010).
ELF4 regulates hematopoietic stem cell quiescence and self-renewal (Lacorazza et al., 2006; Liu et al., 2009). ELF4 also regulates self-renewal of brain-tumor stem cells (Bazzoli et al., 2012).
In addition, ELF4 plays an important role in osteogenic differentiation and bone development (Kim et al., 2007; Seul et al., 2011; Baek et al., 2012).
It mediates DNA damage response (Morales et al., 2008; Sashida et al., 2011).
ELF4 controls cell senescence in a p53-dependent manner and can also promote cellular transformation through inhibition of the p16 pathway as well (Sashida et al., 2009).
ELF4, a member of the ETS transcription factors, can function as an oncogene in murine cancer models and is overexpressed in various human cancers (Lacorazza and Nimer, 2003; Sashida et al., 2010).
In addition to its oncogenic potential, ELF4 has been proposed to be a candidate tumor suppressor gene on the X chromosome (Seki et al., 2002).

Homology

The transcription factor ELF4 belongs to the ETS family of transcription factors, named after the E26 (E twenty six) leukemogenic chicken virus which contains a gag-myb-ets fusion.
These proteins are characterized by the presence of the ETS domain (a conserved 84 amino acid domain) that binds to a DNA consensus sequence containing a GGAA central core via a winged helix-turn-helix motif.
Based on the homology of the ETS domain, these proteins have been classified in different subfamilies (e.g., Elf, Spi, and Yan).
The ETS domain is highly conserved among family members with the greatest homology of ELF4 with other proteins in the Elf subfamily, which also includes ELF1, NERF1a, and NERF1b. ELF4 does not contain an HLH or pointed protein-protein interaction domain, which is present in members of the ETS, ERG, GABP, and Yan subfamilies (Lacorazza and Nimer, 2003).

Mutations

Note
No known mutations. Fused in hepatocellular carcinoma with the BCORL1 gene.

Implicated in

Acute myeloid leukemia

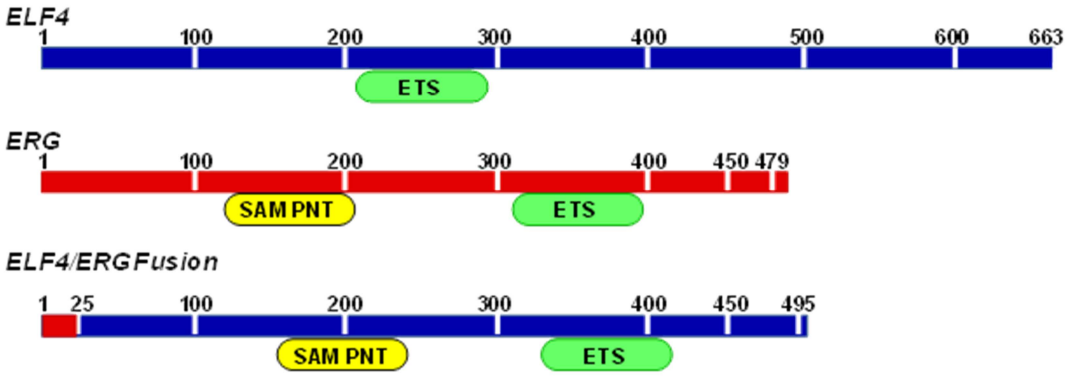
Disease
Acute myeloid leukemia (AML) is the most common acute leukemia in adults. It usually occurs around age 60 with no identifiable cause and it carries a very poor prognosis, with most patients living less than 18 months. AML is a devastating illness with over 13000 new diagnoses and 10000 patients dying annually in the United States. Although select molecular subpopulations of patients are long-term survivors, the overall five-year survival for adults is only 25%. ETS proteins (such as PU.1, Fli-1 and ETS-1) have been shown to play important roles in normal and abnormal hematopoiesis (Lacorazza and Nimer, 2003). ELF1 and ELF4 were expressed in all AML samples, whereas NERF was not. The relative expression of ELF4, but not ELF1, was significantly lower ($P<0.0001$) in AML with t(8;21) and t(15;17) compared with AML with normal karyotype (Fukushima et al., 2003; Ando et al., 2013).

Prognosis
The low relative expression of ELF4, might be part of a gene expression profile characterizing AML with a good prognosis (Fukushima et al., 2003; Ando et al., 2013).

Cytogenetics
A chromosomal translocation has been reported in AML, t(X;21)(q25-26;q22), that generates a fusion transcript between two ETS family members, ELF4 (at Xq26) and ERG (at 21q22). The translocation occurs within intron 1 of ERG isoform 1 (ERG-1) and intron 2 of ELF4 resulting in an in-frame fusion joining exon 2 from ELF4 with exon 2 of ERG (Moore et al., 2006).

Hybrid/Mutated gene
ELF4-ERG. The fusion sequence includes exon 2 of ELF4 and exon 2 of ERG1, with the breakpoint occurring after exon 2 in ELF4 (intron 2) and before exon 2 of ERG1 (intron 1) (Moore et al., 2006).

Abnormal protein
ELF4-ERG (495 amino acids).



Horizontal lines represent amino acid residues for ELF4 and ERG-1. Numbers above each line indicate the number of amino acids at the corresponding position. Boxes below indicate locations of the conserved domains within both protein sequences. The predicted fusion protein would include the first 25 amino acid residues from ELF4 encoded by exon 2 and the last 470 amino acid residues encoded by ERG exons 2-10 resulting in a protein of 495 amino acids. The conserved SAM-PNT and ETS domains from ERG would be included in the fusion while the ETS domain from ELF4 would be excluded.

Oncogenesis

ERG has been associated with other fusion partners, specifically FUS and EWSR1, and implicated in both AML and Ewing's sarcoma. The ELF4-ERG fusion suggests a new role for ELF4 in leukemogenesis and human cancer (Moore et al., 2006), as it provides a strong promoter to express ERG.

In addition, wild-type nucleophosmin (NPM1) inhibits the DNA binding and transcriptional activity of ELF4 on the HDM2 promoter, while a common, leukemia-associated NPM1 mutant protein (Mt-NPM1) enhances these activities of ELF4 (Ando et al., 2013). Moreover, clinical leukemia samples with NPM1 mutations have higher human MDM2 (HDM2) mRNA expression. These data suggest that enhanced HDM2 expression induced by mutant NPM1 may have a role in ELF4-dependent leukemogenesis (Ando et al., 2013).

Ovarian cancer

Disease

Ovarian cancer is the seventh most common cancer in women worldwide and the second leading cause of death among the gynecological malignancies. ELF4 is expressed in a significant proportion of ovarian carcinomas, and in the CAOV3 and SKOV3 ovarian cancer cell lines, but not in normal ovarian surface epithelium (Yao et al., 2007).

Prognosis

High levels of ELF4 expression in ovarian cancer are associated with a poor prognosis (Yao et al., 2007).

Oncogenesis

Manipulating MEF levels in ovarian cancer cell lines alters their behavior; reducing ELF4 levels, using short hairpin RNA expressing vectors, significantly inhibited the proliferation of SKOV3 and CAOV3 cells in culture, and impaired the anchorage-independent growth of CAOV3 cells. Overexpression of ELF4 in SKOV3 cells significantly increased their growth rate, enhanced colony formation in soft agar and promoted tumor formation in nude mice. The oncogenic activity of MEF was further shown by the ability of ELF4 to transform NIH3T3 cells, and induce their tumor formation in nude mice (Yao et al., 2007). Thus, ELF4 is an important regulator of the tumorigenic properties of ovarian cancer cells and could be used a therapeutic target in ovarian cancer (Yao et al., 2007).

Gliomas

Disease

Malignant gliomas represent the most prevalent primary brain tumor in adults and inevitably have a poor prognosis. Despite the implementation of new therapeutic strategies, the median survival of patients with glioblastoma multiforme (GBM), the most aggressive glioma variant, is only 14-16 months and these tumors remain rapidly and uniformly fatal. High-grade gliomas are composed of a heterogeneous population of cells that include many with stem-cell-like properties. The acquisition of stem-like traits might contribute to glioma initiation, growth, and recurrence. ELF4 is highly expressed in both human and mouse glioblastomas and its absence impairs gliomagenesis in a PDGF-driven glioma mouse model (Bazzoli et al., 2012).

Prognosis

High levels of ELF4 expression in gliomas are associated with a poor prognosis (Bazzoli et al., 2012).

Oncogenesis

Modulation of ELF4 levels in both mouse neural stem cells and human glioblastoma cells has a significant impact on neurosphere formation. Thus, ELF4 is a gatekeeper gene that promotes stemness in the pathogenesis of gliomas (Bazzoli et al., 2012).

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PRL2/PTP4A2 Phosphatase Is Important for Hematopoietic Stem Cell Self-Renewal

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ABSTRACT

Hematopoietic stem cell (HSC) self-renewal is tightly controlled by cytokines and other signals in the microenvironment. While stem cell factor (SCF) is an early acting cytokine that activates the receptor tyrosine kinase KIT and promotes HSC maintenance, how SCF/KIT signaling is regulated in HSCs is poorly understood. The protein tyrosine phosphatase 4A (PTP4A) family (aka PRL [phosphatase of regenerating liver] phosphatases), consisting of PTP4A1/PRL1, PTP4A2/PRL2, and PTP4A3/PRL3, represents an intriguing group of phosphatases implicated in cell proliferation and tumorigenesis. However, the role of PTP4A in hematopoiesis remains elusive. To define the role of PTP4A in hematopoiesis, we analyzed HSC behavior in *Ptp4a2* (*Prl2*) deficient mice. We found that *Ptp4a2* deficiency impairs HSC self-renewal as revealed by serial bone marrow transplantation assays. Moreover, we observed that *Ptp4a2* null hematopoietic stem and progenitor cells (HSPCs) are more quiescent and show reduced activation of the AKT and ERK signaling. Importantly, we discovered that the ability of PTP4A2 to enhance HSPC proliferation and activation of AKT and ERK signaling depends on its phosphatase activity. Furthermore, we found that PTP4A2 is important for SCF-mediated HSPC proliferation and loss of *Ptp4a2* decreased the ability of oncogenic KIT/D814V mutant in promoting hematopoietic progenitor cell proliferation. Thus, PTP4A2 plays critical roles in regulating HSC self-renewal and mediating SCF/KIT signaling. STEM CELLS *2014;32:1956–1967*

INTRODUCTION

In order to maintain hematopoietic homeostasis throughout the life of an animal, the hematopoietic stem cell (HSC) pool must be maintained through the process of self-renewal [1]. HSC self-renewal requires the integration of survival and proliferation signals to maintain an undifferentiated state. This demands a complex crosstalk between extrinsic signals from the microenvironment and the cell-intrinsic regulators of HSCs [2, 3]. Stem cell factor (SCF) is a dimeric molecule that exerts its biological functions by binding to and activating the receptor tyrosine kinase KIT [4]. KIT is highly expressed in HSCs and SCF/KIT signaling plays a critical role in HSC maintenance [5–14]. SCF is an early acting cytokine that promotes HSC proliferation and survival [5–8]. Studies in mice with either partial or complete loss-of-function KIT mutations have revealed severe hematopoietic deficiencies of multiple lineages as well as an important role of KIT in sustaining rapidly cycling HSCs post-transplantation when competing with wild-type HSCs [9–14]. Despite the wealth of knowledge on SCF/KIT signaling, it is poorly understood how KIT signaling is regulated in HSCs [4].

Gain-of-function mutations in KIT receptor in humans are associated with gastrointestinal stromal tumors, systemic mastocytosis, and acute myelogenous leukemia (AML) [15–17]. These mutations results in altered substrate recognition and constitutive tyrosine autophosphorylation leading to promiscuous and constitutive signaling [18–20]. Consequently, cell lines and primary bone marrow (BM) cells that express the oncogenic KIT mutant demonstrate ligand-independent proliferation in vitro and myeloproliferative disease (MPD) in vivo [18–22]. However, the intracellular signals that contribute to mutant KIT-induced MPD are not known.

The PRL (phosphatase of regenerating liver) phosphatases constitute a novel class of small, prenylated phosphatases (PRL1, 2, and 3) that share a high degree (>76%) of sequence identity [23–25]. The PRLs are relatively small proteins of about 20 kDa. In addition to the phosphatase domain, there are no regulatory domains except that all PRLs contain a consensus C-terminal prenylation motif CaaX, which is important for their localization to the plasma membrane and early endosomal compartments [23–25]. This family of phosphatases is also known as protein tyrosine phosphatase 4As (PTP4As). *PTP4A1/PRL1* was

originally identified as an immediate early gene induced during liver regeneration after partial hepatectomy [23]. Subsequently, *PTP4A1/PRL1* as well as the closely related *PTP4A2/PRL2* and *PTP4A3/PRL3* was found to be elevated in numerous tumor cell lines, and cells expressing high levels of PTP4As exhibit enhanced proliferation and anchorage-independent growth [24–29]. Unlike most protein phosphatases that counteract the activity of protein kinases, the PTP4As play a positive role in signaling and possess oncogenic properties [30, 31]. Consistent with their oncogenic potential, we recently revealed that PTP4A2/PRL2 promotes placenta development by downregulating PTEN, leading to AKT activation [32].

Ptp4a2/Prl2 is located on human chromosome 1p35 [30], a region often rearranged or amplified in malignant lymphoma and B-cell chronic lymphocytic leukemia [33, 34]. While *Ptp4a2/Prl2* mRNA is highly expressed in pediatric acute myeloid leukemia cells [35, 36], its role in normal and malignant hematopoiesis is largely unknown. Here, we report a functional requirement of PTP4A2/PRL2 in HSC self-renewal. We further demonstrate that PTP4A2/PRL2 is an important mediator of SCF/KIT signaling in HSCs.

Materials and Methods

Mice

The generation of *Ptp4a2/Prl2* knockout mice (*Ptp4a2*$^{-/-}$, C57BL6/129P2 mixed background) has been described previously [32]. *Ptp4a2/Prl2* knockout mice were backcrossed with C57BL6 mice for at least eight generations. Wild-type C57BL/6 (CD45.2$^+$), B6.SJL (CD45.1$^+$), and F1 mice (CD45.2$^+$ CD45.1$^+$) were purchased from the Jackson Laboratories. All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols, and kept in Thorensten units with filtered germ-free air.

Flow Cytometry

Flow cytometry analysis of hematopoietic stem and progenitor cells (HSPCs) was performed as described previously [37, 38]. Murine HSPCs were identified and evaluated by flow cytometry using a single-cell suspension of bone marrow mononuclear cells (BMMCs). Hematopoietic stem and progenitors are purified based upon the expression of surface markers: long-term (LT)-HSC (Lin$^-$Sca1$^+$Kit$^+$CD34$^-$CD48$^-$ CD150$^+$), short-term (ST)-HSC (Lin$^-$Sca1$^+$Kit$^+$CD34$^+$CD48$^+$ CD150$^+$), multipotent progenitor cell (MPP) (Lin$^-$Sca1$^+$Kit$^+$ CD34$^+$CD48$^+$CD150$^-$), common myeloid progenitor (Lin$^-$Sca1$^-$IL7R$^-$Kit$^+$FcγRII/III^{low}CD34^{high}), granulocyte-macrophage progenitor (Lin$^-$Sca1$^-$IL7R$^-$Kit$^+$FcγRII/III^{high}CD34^{high}), megakaryocyte-erythroid progenitor cell (MEP) (Lin$^-$Sca1$^-$IL7R$^-$Kit$^+$FcγRII/III^{low}CD34^{low}), and common lymphoid progenitor (Lin$^-$IL7R$^+$Sca1^{low}Kit^{low}). BMMCs were obtained from both tibias and femurs by flushing cells out of the bone using a syringe and Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FBS). Cells were first stained with a lineage (Lin) cocktail of antibodies from BD Biosciences (biotinylated anti-mouse antibodies directed against CD3e, CD11b, CD45R/B220, Gr-1, and Ter119) as well as Sca-1 PE and c-KIT APC (BD Biosciences, San Jose, CA; http://www.bdbiosciences.com/), and a streptavidin Cychrome conjugate (BD Biosciences, San Jose, CA; http://www.bdbiosciences.com). c-KIT-APC, Sca-1-PE-Cy7, Flt3-PE, CD34-FITC, and streptavidin APC-Cy7 were used for analysis using a FACSLSR II cytometer (BD Biosciences, San Jose, CA; http://www.bdbiosciences.com/). FITC-CD41, FITC-CD48, and FITC-CD34 were purchased from eBioscience and PE-CD150 purchased from Biolegend for SLAM marker analysis. All other antibodies were from BD Biosciences. For immunophenotypic analysis, approximately 3 × 10^6 BMMCs were stained with antibodies for 30 minutes on ice in dark. Nuclear staining of Ki67 was assessed using an FITC-anti-human Ki67 antibody (BD, Biosciences, San Jose, CA; http://www.bdbiosciences.com/) and fixation and permeabilization solutions from BD Biosciences. Experiments were performed on FACSAria and FACSLSR II cytometers (BD Biosciences, San Jose, CA; http://www.bdbiosciences.com/home.jsp) and analyzed using the FlowJo Version 9.3.3 software (TreeStar, Ashland, OR; http://www.treestar.com/).

BM Transplantation

For the competitive repopulation assays, we injected 5 × 10^5 BMMCs from wild-type and *Ptp4a2* null mice (CD45.2$^+$) plus 5 × 10^5 competitor BM cells (CD45.1$^+$) into lethally irradiated F1 mice (CD45.1$^+$CD45.2$^+$). Peripheral blood was obtained by tail vein bleeding every month, the RBC lysed, and the Peripheral blood mononuclear cells (PBMCs) stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. Sixteen weeks following transplantation, we harvested BM cells from mice reconstituted with wild-type or *Ptp4a2* null BM cells and transplanted 3 × 10^6 BM cells into lethally irradiated F1 mice (CD45.1$^+$CD45.2$^+$).

For HSC transplantation, we injected 200 CD48$^-$CD150$^+$KSL cells from wild-type or *Ptp4a2* null mice (CD45.2$^+$) plus 3 × 10^5 competitor BM cells (CD45.1$^+$) into lethally irradiated F1 mice (CD45.1$^+$CD45.2$^+$). Peripheral blood was obtained by retro-orbital eye bleeding every month, the RBC lysed, and the PBMCs stained with anti-CD45.2 FITC and anti-CD45.1 PE, and analyzed by flow cytometry. Sixteen weeks following transplantation, we harvested BM cells from recipient mice and performed flow cytometry analysis to evaluate HSC repopulating capability.

Homing Assays

The homing ability of wild-type and *Ptp4a2* null HSPCs was analyzed in irradiated recipient mice. 1 × 10^7 wild-type or *Ptp4a2* null BM cells (CD45.2$^+$) were injected into lethally irradiated recipient (CD45.1$^+$) mice. BM cells were harvested 18 hours following injection and donor-derived cells were evaluated by flow cytometry as CD45.2$^+$ cells, simultaneously identifying Lin$^-$Sca1$^+$CD150$^+$ cells.

Stem and Progenitor Cell Assays

Clonogenic progenitors were determined in methylcellulose medium (MethoCult GF M3234, Stem Cell Technologies, Vancouver, Canada; http://www.stemcell.com/) with cytokines (SCF, TPO, EPO, IL-3, and GM-CSF) using 2 × 10^4 BMMCs per well (six-well plate). Colonies were scored after 7 days of the initial culture, and all cells were collected and washed twice in phosphate-buffered saline. Subsequently cells were cultured at 2 × 10^4 per well in the same medium. Colony scoring and replating were repeated every 7 days for at least two times, or until no colonies were observed in the cultures.

Immunoblotting

Cells were washed with ice-cold phosphate-buffered saline, and lysed on ice for 30 minutes in lysis buffer (50 mM

Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, and 10% glycerol) supplemented with protease inhibitor (Roche, Basel, Switzerland; http://www.roche.com/) and phosphatase inhibitor (Roche, Basel, Switzerland; http://www.roche.com/). Cell lysates were cleared by centrifugation at 13,200 rpm for 10 minutes, and boiled with Laemmli (SDS)-sample buffer, separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane, and immunoblotted with pAKT (Cell Signaling), AKT (Cell Signaling, Danvers, MA; https://www.cellsignal.com/), pERK1/2 (Cell Signaling, Danvers, MA; https://www.cellsignal.com/), ERK1/2 (Cell Signaling), PTEN (Cell Signaling, Danvers, MA; https://www.cellsignal.com/), PTP4A2/PRL2 (a generous gift from Dr. Qi Zeng), and β-Actin (Santa Cruz, Dallas, Texas; http://www.scbt.com/) followed by incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were developed by the enhanced chemiluminescence technique using the SuperSignal West Pico Chemiluminescent substrate (Fisher Scientific, Hampton, NH; http://www.fishersci.com/) or SuperSignal West Femto Maximum Sensitivity Chemiluminescent Substrate (Fisher Scientific, Hampton, NH; http://www.fishersci.com/). Data shown are representation of multiple repeat experiments.

Statistical Analysis

We used either Student's *t* test or two-way analysis of variance to determine statistical significance. *, $p < .05$; **, $p < .01$; ***, $p < .001$; ns, not significant.

Results

Ptp4a2 Deficiency Results in Inefficient Hematopoiesis

To investigate the role of PTP4A2 in hematopoiesis, we analyzed the peripheral blood and BM of 8–12-week-old wild-type and *Ptp4a2*$^{-/-}$ mice. *Ptp4a2*$^{-/-}$ mice show decreased white blood cell, neutrophil, and lymphocyte counts, whereas hemoglobin levels are normal in the peripheral blood (Supporting Information Fig. S1A). While *Ptp4a2* null mice show decreased body size as we previously reported (Supporting Information Fig. S1B) [32], the BM cellularity is normal when normalized to total body weight (Supporting Information Fig. S1C). We also found that *Ptp4a2*$^{-/-}$ mice have smaller spleen and thymus compared with wild-type mice (Supporting Information Fig. S2). These data indicate that PTP4A2 plays an important role in hematopoiesis.

Ptp4a2 Null Mice Have Normal Number of Immunophenotypic HSCs

While *Ptp4a1* and *Ptp4a2* are broadly expressed in adult tissues including liver [25, 39, 40], the expression pattern of these *Ptp4as* in the hematopoietic system is unknown. We examined the expression of *Ptp4a1*, *Ptp4a2*, and *Ptp4a3* during different stages of HSC differentiation using real-time RT-PCR assays. *Ptp4a1* and *Ptp4a2* are ubiquitously expressed in the hematopoietic compartment, whereas *Ptp4a3* expression is most pronounced in MEPs. *Ptp4a2* is highly expressed in hematopoietic cells and its expression is enriched in differentiated cells compared to LT-HSCs and committed progenitor cells, suggesting that it could play an important role in lineage commitment (Fig. 1A). We then examined the number of primitive hematopoietic stem/progenitor cells in the BM of *Ptp4a2*$^{-/-}$ mice. Loss of *Ptp4a2* slightly increased the frequency (*, $p < .05$, $n = 9$; Fig. 1B), but not the absolute number of Kit$^+$Sca1$^+$Lin$^-$ cells (KSLs) compared to wild-type mice (Fig. 1C); however, *Ptp4a2* deficiency neither affects the frequency nor the absolute number of LT-HSCs based on SLAM and CD34 expression (Fig. 1D, 1E). Thus, loss of *Ptp4a2* does not affect immunophenotypic HSCs.

Ptp4a2 Deficiency Impairs the Long-Term Repopulating Ability of HSCs

To examine whether *Ptp4a2* deficiency affects HSC function, we performed serial competitive BM transplantation assays. Equal numbers of donor and competitor BMMCs were transplanted into lethally irradiated recipient mice. Sixteen weeks after primary transplantation, the repopulating ability of *Ptp4a2* null cells was significantly lower than wild-type cells (**, $p < .01$, $n = 7$; Fig. 2A). Moreover, we found that the percentage of donor-derived T cells was significantly decreased in mice transplanted with *Ptp4a2* null cells than that with wild-type cells (11.0% $\pm$ 1.9% vs. 4.1% $\pm$ 1.7% at 16 weeks, Fig. 2B). Analysis of the BM revealed a striking reduction in the number of phenotypically defined HSCs in the recipients repopulated with *Ptp4a2* null BM cells (**, $p < .01$, $n = 5$; Fig. 2C). We then transplanted 3×10^6 BMMCs isolated from the primary recipient mice repopulated with wild-type or *Ptp4a2* null cells into lethally irradiated secondary recipients. Sixteen weeks after transplantation, *Ptp4a2* null cells continued to show decreased repopulating ability (***, $p < .001$, $n = 7$; Fig. 2D).

To further establish that the self-renewal defect is HSC intrinsic, we purified wild-type and *Ptp4a2* null LT-HSCs (CD48$^-$CD150$^+$KSLs) and transplanted 200 of each into lethally irradiated recipient mice along with 300,000 wild-type competitor BMMCs. In this context, *Ptp4a2* null HSCs exhibited a substantially lower contribution to peripheral blood production compared to control HSCs (***, $p < .001$, $n = 7$; Fig. 2E), demonstrating that Ptp4a2 plays an important role in HSC maintenance and loss of *Ptp4a2* impairs HSC self-renewal.

The decreased self-renewal of *Ptp4a2* null HSCs in transplantation assays could be due to abnormalities in homing following transplantation. To determine whether *Ptp4a2* null BM cells are defective in homing, we performed homing assays and observed similar numbers of donor-derived Lin$^-$Sca-1$^+$Slam$^+$ cells from the BM of recipient mice repopulated with wild-type or *Ptp4a2* null BM cells (Supporting Information Fig. S3A), suggesting the absence of homing defects. The impaired HSC self-renewal in *Ptp4a2* null mice could also be due to increased apoptosis. Therefore, we evaluated HSC survival by Annexin-V staining and observed no significant difference in the number of apoptotic HSCs (Annexin-V$^+$/PI$^-$CD48$^-$KSLs) in the absence of cytokines (Supporting Information Fig. S3B). Thus, the impaired self-renewal of *Ptp4a2* null HSC is likely due to an inability to properly self-renew, rather than homing defects or an increase in apoptosis.

PTP4A2 Promotes HSPC Proliferation

To assess the effect of *Ptp4a2* deficiency on proliferation, we stained *Ptp4a2* null HSPCs with proliferation marker Ki67. While we detected normal number of Ki67 negative LT-HSCs, we found more Ki67-negative MPPs than normal (**, $p < .01$, $n = 6$; Fig. 3A), indicating *Ptp4a2* null MPPs are more

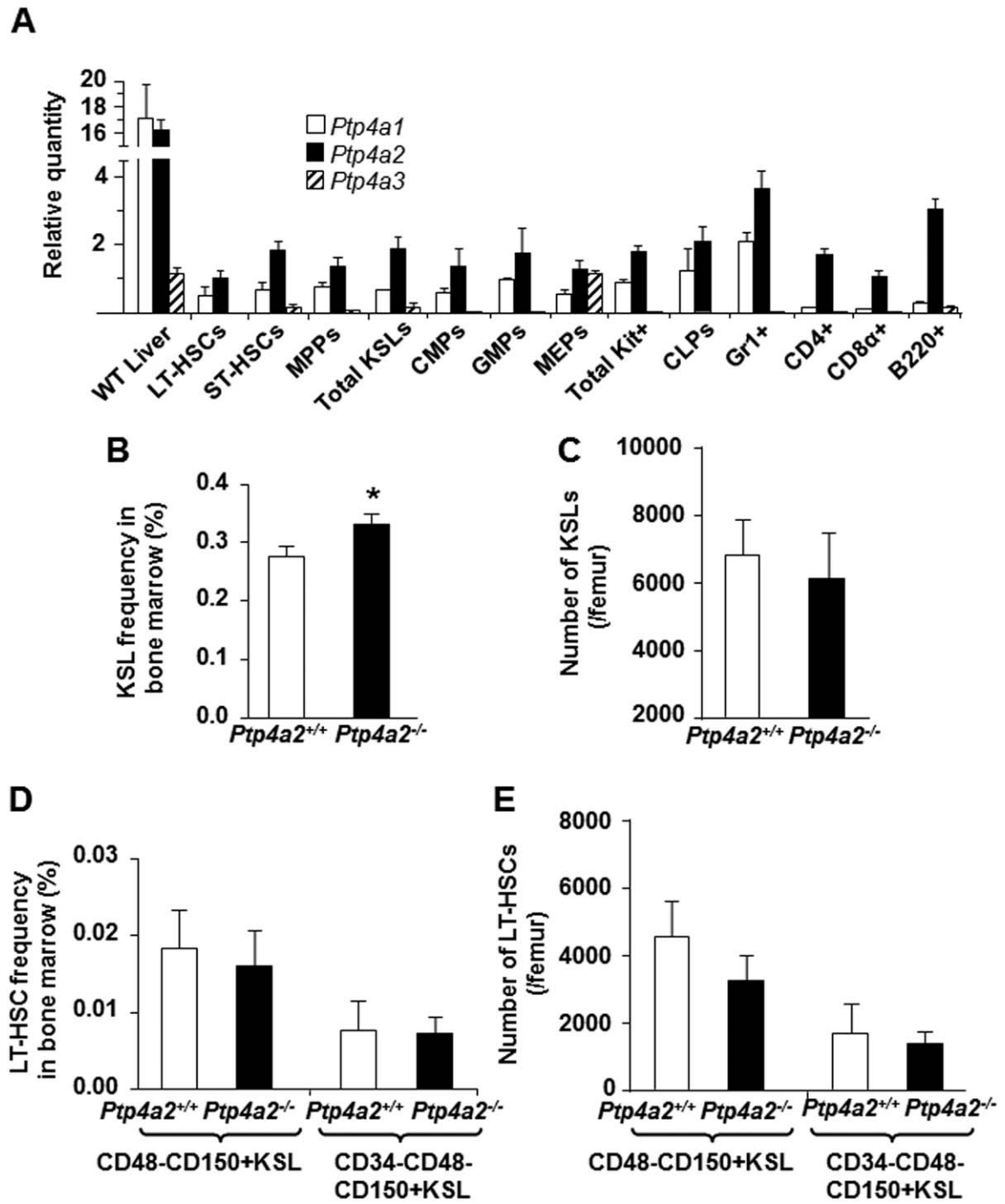

Figure 1. *Ptp4a2* null mice have normal number of immunophenotypic hematopoietic stem cells. **(A):** Real-time RT-PCR analysis of *Ptp4a* mRNAs in LT-HSCs, ST-HSCs, and representative committed progenitors and differentiated cells. Data shown are the mean values ± SD ($n = 3$ biological replicates). **(B):** The frequency of $Kit^+Sca1^+Lin^-$ cells (KSLs) was quantified by flow cytometry. Data shown are the mean percentage (±SD) of KSL cells in the bone marrow (*, $p < .05$, $n = 9$). **(C):** The absolute number of KSLs per femur is shown. Graphs represent the mean ± SD ($n = 9$). **(D):** The frequency of LT-HSCs ($CD34^-CD48^-CD150^+$KSLs) was defined by flow cytometry analysis of SLAM cell surface markers. Data shown are the mean percentage (±SD) of LT-HSCs in the bone marrow ($n = 9$). **(E):** The absolute number of LT-HSCs per femur is shown. Graphs represent the mean ± SD ($n = 9$). Abbreviations: CLPs, common lymphoid progenitors; CMPs, common myeloid progenitors; GMPs, granulocyte-macrophage progenitors; LT-HSCs, long-term hematopoietic stem cells; MEPs, megakaryocyte-erythroid progenitors; MPPs, multi-potential progenitors; ST-HSCs, short-term HSCs.

quiescent and less proliferative. We then performed methylcellulose colony-forming unit (CFU) assays to quantify myeloid progenitor cells. While *Ptp4a2* deficiency does not affect the total number of colonies formed, *Ptp4a2* null BM cells formed significantly fewer immature GEMM (granulocyte, erythrocyte, monocyte, megakaryocyte) colonies compared with wild-type cells (*, $p < .05$, $n = 3$; Fig. 3B). Consistent with the cell cycle data, *Ptp4a2* null BMMCs show decreased replating potential

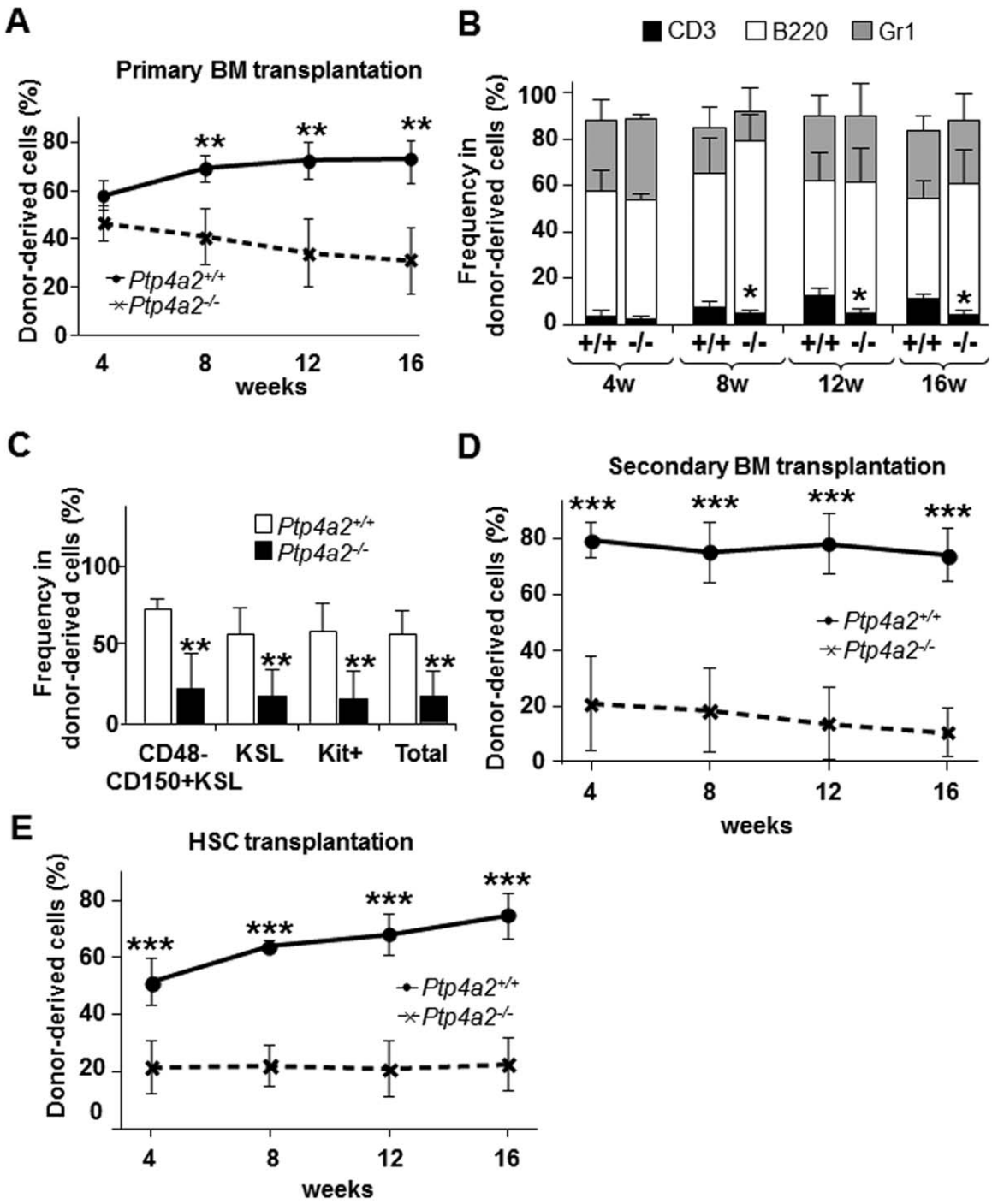

Figure 2. *Ptp4a2* deficiency impairs the long-term repopulating ability of HSCs. **(A):** Lethally irradiated recipient mice ($CD45.1^+$ and $CD45.2^+$) were transplanted with 5×10^5 bone marrow mononuclear cells (BMMCs) from wild-type and *Ptp4a2* null mice ($CD45.2^+$) plus 5×10^5 competitor cells ($CD45.1^+$) in competitive repopulation assays. Graph shows the mean percentage (±SD) of donor-derived ($CD45.2^+$) cells in the peripheral blood post-transplantation, measured at monthly intervals (**, $p < .01$, $n = 7$). **(B):** Contribution of donor-derived cells ($CD45.2^+$) from A, for myeloid cells ($Gr1^+$), B cells ($B220^+$), and T cells ($CD3^+$) in peripheral blood from recipient mice at different time points after transplantation (*, $p < .05$, $n = 7$). **(C):** Quantification of donor-derived ($CD45.2^+$) HSC frequency in the bone marrow of primary recipient mice 18 weeks following transplantation by three phenotypic definitions. Mean ± SD values are shown (**, $p < .01$, $n = 5$). **(D):** Contribution of wild-type and *Ptp4a2* null BMMCs to recipient mouse peripheral blood in secondary competitive transplants, measured at monthly intervals (***, $p < .001$, $n = 7$). **(E):** Lethally irradiated recipient mice ($CD45.1^+$ and $CD45.2^+$) were transplanted with 200 LT-HSCs ($CD48^-CD150^+$KSLs) from wild-type and *Ptp4a2* null mice ($CD45.2^+$) plus 3×10^5 competitor cells ($CD45.1^+$) in competitive repopulation assays. Graph shows the mean percentage (±SD) of donor-derived ($CD45.2^+$) cells in the peripheral blood post-transplantation, measured at monthly intervals (***, $p < .001$, $n = 7$). Abbreviations: BM, bone marrow; HSC, hematopoietic stem cell; KSL, $Kit^+Sca1^+Lin^-$ cell.

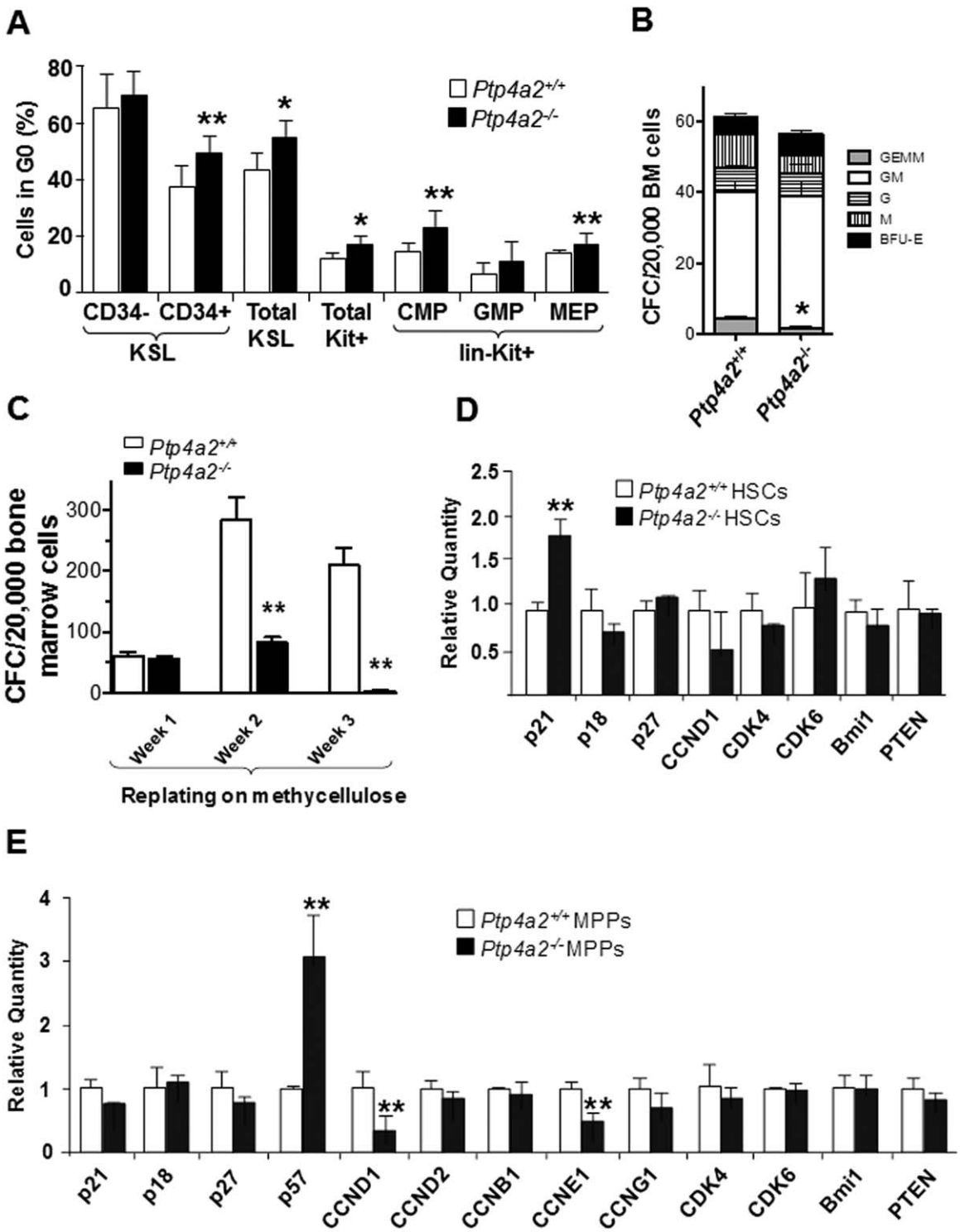

Figure 3. PTP4A2 regulates hematopoietic stem and progenitor cell proliferation. **(A):** Cell-cycle analysis of hematopoietic stem and progenitor cells was performed by staining with DAPI and Ki67 and analyzed by fluorescence-activated cell sorting. Data shown are the mean values ± SD (**, $p < .01$, $n = 6$). **(B):** Myeloid progenitors were quantified by methylcellulose culture using bone marrow mononuclear cells (BMMCs) from wild-type and *Ptp4a2* null mice (*, $p < .05$, $n = 3$). **(C):** Serial replating studies. Myeloid progenitors were quantified by methylcellulose culture using BMMCs from wild-type and *Ptp4a2* null mice. The methylcellulose cultures were serially replated, weekly, for 3 weeks. Mean values (±SD) are shown (**, $p < .01$, $n = 3$). **(D, E):** *Ptp4a2* deficiency results in upregulation of cell cycle regulators in hematopoietic stem cells (HSCs) (D) and MPPs (E). Real-time RT-PCR analysis of some known HSC regulators in LT-HSCs and MPPs. Data shown are the mean values ± SD (**, $p < .01$, $n = 3$ biological replicates). Abbreviations: CMPs, common myeloid progenitors; GMPs, granulocyte-macrophage progenitors; KSL, $Kit^+Sca1^+Lin^-$ cell; MEPs, megakaryocyte-erythroid progenitors; MPPs, multipotential progenitors.

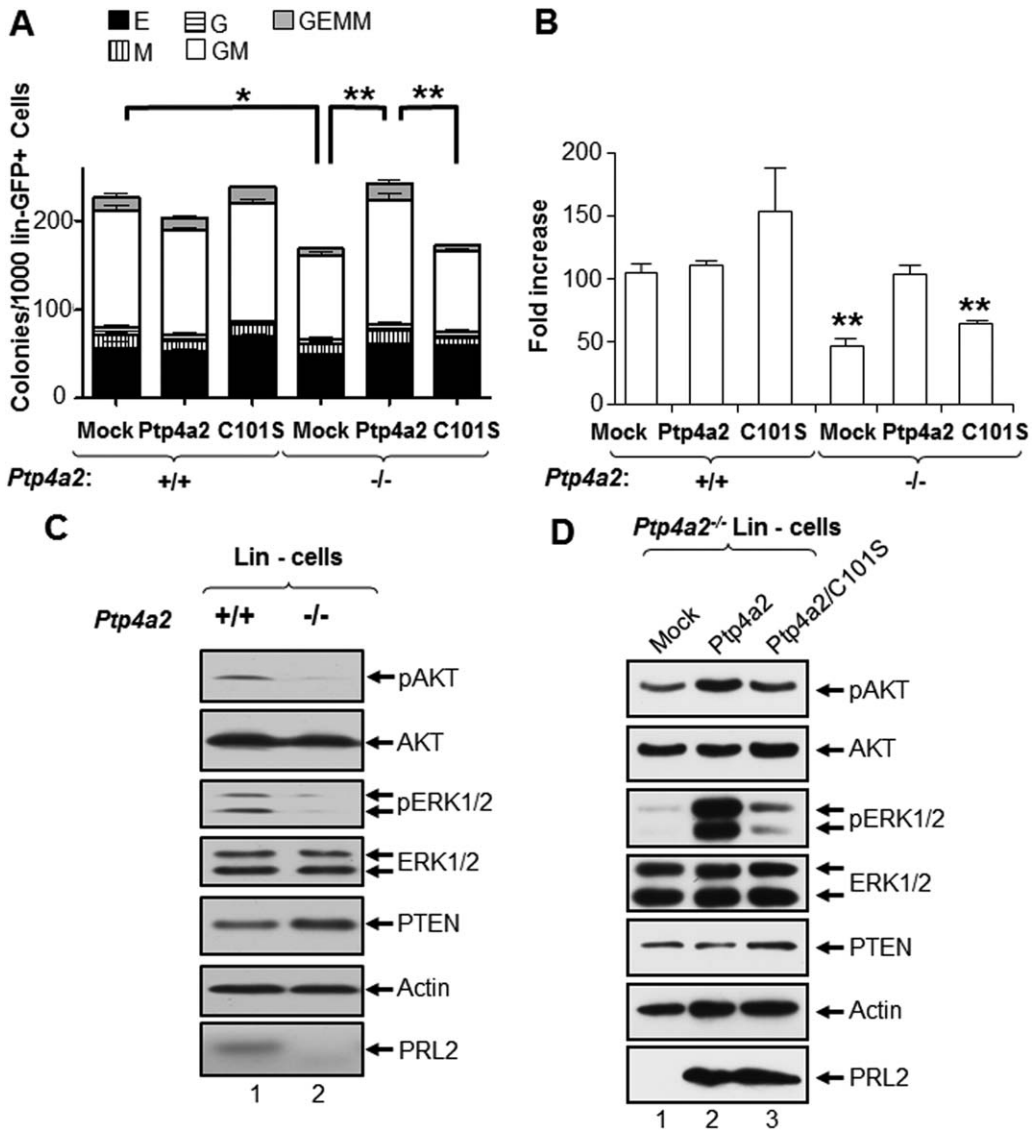

Figure 4. The ability of PTP4A2 to enhance hematopoietic progenitor cell proliferation depends on its phosphatase activity. **(A):** Myeloid progenitors were quantified by methylcellulose culture using wild-type and *Ptp4a2* null Lin− cells transduced with retroviruses expressing the WT or the mutant form (Ptp4a2/C101S) of Ptp4a2. Data are means ± SD (**, $p < .01$, $n = 3$ independent experiments). **(B):** Proliferation of wild-type and *Ptp4a2* null Lin^- cells expressing the WT or the mutant form (Ptp4a2/C101S) of Ptp4a2 in response to SCF stimulation. Data are means ± SD (**, $p < .01$, $n = 3$ independent experiments). **(C):** Immunoblot analysis of AKT and ERK phosphorylation in freshly purified wild-type and *Ptp4a2* null Lin^- cells. *, $p < .05$ compared with wild-type cells by one-way analysis of variance (ANOVA) and Bonferroni post hoc test, $n = 3$ independent experiments. Representative Western blot analysis of indicated proteins is shown. **(D):** Immunoblot analysis of AKT and ERK phosphorylation in *Ptp4a2* null Lin^- cells expressing the WT or the mutant form (Ptp4a2/C101S) of Ptp4a2. *, $p < .05$ compared with mock transduced cells by one-way ANOVA and Bonferroni post hoc test, $n = 3$ independent experiments. Representative Western blot analysis of indicated proteins is shown.

compared to wild-type cells (**, $p < .01$, $n = 3$; Fig. 3C), further demonstrating that hematopoietic progenitor cells show decreased proliferation in the absence of *Ptp4a2*. To decipher the molecular mechanisms underlying the HSPC proliferation defects seen in the *Ptp4a2* null mice, we examined the expression of several known HSC regulators using real-time RT-PCR assays. Several negative regulators of cell cycle, including *p21* and *p57*, are upregulated in *Ptp4a2* null HSCs and MPPs, respectively (**, $p < .01$, $n = 3$; Fig. 3D, 3E). In addition, the expression of *Cyclin D1* (*CCND1*) and *Cyclin E1* (*CCNE1*) is downregulated in MPPs (**, $p < .01$, $n = 3$; Fig. 3E). We further confirmed the changes in p57 and CCND1 expression in MPPs by flow cytometry (Supporting Information Fig. S4).

The Ability of PTP4A2 to Enhance Hematopoietic Progenitor Cell Proliferation Depends on Its Phosphatase Activity

While we showed that PTP4A2 is important for hematopoietic progenitor cell proliferation, whether this effect depends on its phosphatase activity is not clear. To this end, we overexpressed wild-type or the catalytic inactive mutant form of *Ptp4a2* (*Ptp4a2/C101S*) in Lin^- cells isolated from wild-type and *Ptp4a2* null mice and performed CFU assay [41]. While

both wild-type and *Ptp4a2* null mononuclear cells form similar number of colonies in CFU assays (Fig. 3B), *Ptp4a2* null Lin-Sca1+ cells formed significantly fewer colonies compared with wild-type cells following retroviral transduction (*, $p < .05$, $n = 3$; Fig. 4A). Lin-Sca1+ cells are enriched for HSPCs and we showed that *Ptp4a2* null progenitor cells are less proliferative (Fig. 3A). Therefore, the difference between the two CFU assays we observed is likely due to distinct composition of cells used in the experiments. Ectopic expression of wild-type or mutant *Ptp4a2* did not affect the colony-forming potential of wild-type cells. However, we found that overexpressing wild-type *Ptp4a2*, but not the mutant form, significantly increase the colony formation of *Ptp4a2* null cells (**, $p < .01$, $n = 3$; Fig. 4A). We also overexpressed wild-type or the mutant form of *Ptp4a2* in Lin^- cells isolated from wild-type and *Ptp4a2* null mice and monitored their proliferation. Consistent with data in the CFU assays (Fig. 4A), mock-transduced *Ptp4a2* null Lin^- cells show decreased proliferation compared with that of mock-transduced wild-type cells (**, $p < .01$, $n = 3$; Fig. 4B). Wild-type *Ptp4a2* transduced *Ptp4a2* null cells showed enhanced proliferation compared with mock and mutant *Ptp4a2* transduced cells (**, $p < .01$, $n = 3$; Fig. 4B).

To decipher the molecular mechanisms underlying the HSPC proliferation defects seen in the *Ptp4a2* null mice, we examined the activation of AKT and ERK signaling in freshly purified Lin^- cells. We observed that the levels of pAKT and pERK1/2 are significantly lower in *Ptp4a2* null Lin− cells compared with those in wild-type cells (Fig. 4C). To investigate the mechanism by which *Ptp4a2* deficiency attenuates AKT and ERK activation, we analyzed PTEN (phosphatase and tensin homolog deleted on chromosome 10) expression in both wild-type and *Ptp4a2* null cells. PTEN is an antagonist of PI3K signaling pathway [42]. In addition, PTEN has also been shown to negatively regulate the activation of the ERK pathway [42]. We previously reported that in placenta, *Ptp4a2* deficiency led to elevated PTEN expression [32]. To examine whether this is also the case in HSPCs, we measured PTEN protein level in wild-type and *Ptp4a2* null Lin− cells. As shown in Figure 4C, PTEN expression was 1.4 ± 0.15-fold higher in *Ptp4a2* null samples, consistent with our previous observation in placenta [32]. Therefore, impaired AKT and ERK activation in *Ptp4a2* null Lin− cells are likely due to increased PTEN level. Furthermore, we found that expression of wild-type *Ptp4a2*, but not the catalytically inactive mutant *Ptp4a2*, significantly augments pAKT and pERK1/2 levels in *Ptp4a2* null Lin^- cells (Fig. 4D and Supporting Information Fig. S5), demonstrating that the ability of PTP4A2 to promote hematopoietic progenitor cell proliferation and activation of AKT and ERK signaling depends on its phosphatase activity.

PTP4A2 Is Important for SCF/KIT Signaling in HSPCs

Given that *Ptp4a2* null BMMCs show decreased colony formation in serial replating assays, PTP4A2 may play an important role in cytokine signaling. To test this hypothesis, we cultured both wild-type and *Ptp4a2* null Kit^+ cells in the presence or absence of cytokines, including SCF, G-CSF, and IL-3, and monitored their proliferation. While wild-type and *Ptp4a2* null Kit^+ cells exhibited similar proliferative behaviors in response to G-CSF or IL-3 stimulation, *Ptp4a2* null hematopoietic progenitor cells showed decreased proliferation in response to SCF (*, $p < .05$, $n = 3$; Fig. 5A). In CFU assays, *Ptp4a2* null KSL cells formed significantly fewer colonies compared with wild-type KSL cells in response to SCF stimulation (**, $p < .01$, $n = 3$; Fig. 5B). Importantly, *Ptp4a2* null KSL cells showed marked reduction of GEMM colony formation in response to decreased concentration of SCF compared with wild-type KSL cells, demonstrating that PTP4A2 is essential for the proliferation of hematopoietic progenitor cells following SCF stimulation. We then examined whether *Ptp4a2* deficiency affects HSPC survival in the absence of cytokine. As shown in Figure 5C, *Ptp4a2* null Kit^+ cells showed decreased cell counts compared with wild-type cells (*, $p < .05$, $n = 3$), indicating that PTP4A2 is essential for HSPC survival in the absence of cytokine. Furthermore, we observed that the levels of pAKT and pERK1/2 are also significantly lower in *Ptp4a2* null Kit^+ cells compared with those in wild-type cells in the basal state (Fig. 5D). Stimulation of HSPCs with SCF can activate several signaling pathways through KIT, including PI3K/AKT and ERK1/2 pathways [4–6]; therefore, it is possible that *Ptp4a2* deficiency impairs the activation of these signaling pathways in response to SCF stimulation. This is indeed the case, as *Ptp4a2* null cells displayed decreased activation of both pAKT and pERK1/2 at 30 minutes following SCF stimulation (Fig. 5D), demonstrating that PTP4A2 is important for sustaining SCF signaling in HSPCs.

PTP4A2 Is Important for Oncogenic KIT Signaling in Hematopoietic Progenitor Cells

Hematopoietic cell lines and primary BM cells expressing the oncogenic KIT/D814V mutant display ligand-independent proliferation in vitro and MPD in vivo [18–22]. Based on our finding that PTP4A2 is important for SCF/KIT signaling in HSPCs, we speculated that PTP4A2 may also enhance KIT/D814V-mediated ligand-independent hyperproliferation and activation of signaling pathways. To test this, we introduced wild-type KIT and KIT/D814V mutant into Lin^- cells purified from wild-type and *Ptp4a2* null mice and performed serial replating assays. In primary (*, $p < .05$, $n = 3$), secondary (**, $p < .01$, $n = 3$), and tertiary replating experiments (***, $p < .001$, $n = 3$), *Ptp4a2* null Lin^- cells expressing KIT/D814V formed significantly fewer colonies compared with wild-type Lin^- cells expressing the KIT mutant (Fig. 6A). Both wild-type and *Ptp4a2* null cells expressing wild-type KIT did not form any colonies in the tertiary replating experiments (Fig. 6A). To determine whether PTP4A2 is essential for KIT/D814V-mediated ligand-independent growth, we cultured *Ptp4a2* wild-type and null cells expressing wild-type or KIT/D814V in the absence of cytokine or the presence of SCF. As shown in Figure 6B (**, $p < .01$, $n = 3$), *Ptp4a2* deficiency significantly decreased the ability of KIT/D814V to promote cell proliferation both in the absence and presence of SCF. Furthermore, we observed decreased phosphorylation of AKT and ERK1/2 in *Ptp4a2* null cells expressing KIT/D814V mutant compared with wild-type cells (Fig. 6C). Thus, PTP4A2 is essential for KIT/D814V-mediated hematopoietic progenitor cell hyperproliferation and activation of signaling pathways.

Discussion

The PTP4A family of phosphatases, consisting of PTP4A1, PTP4A2, and PTP4A3, represents an intriguing group of

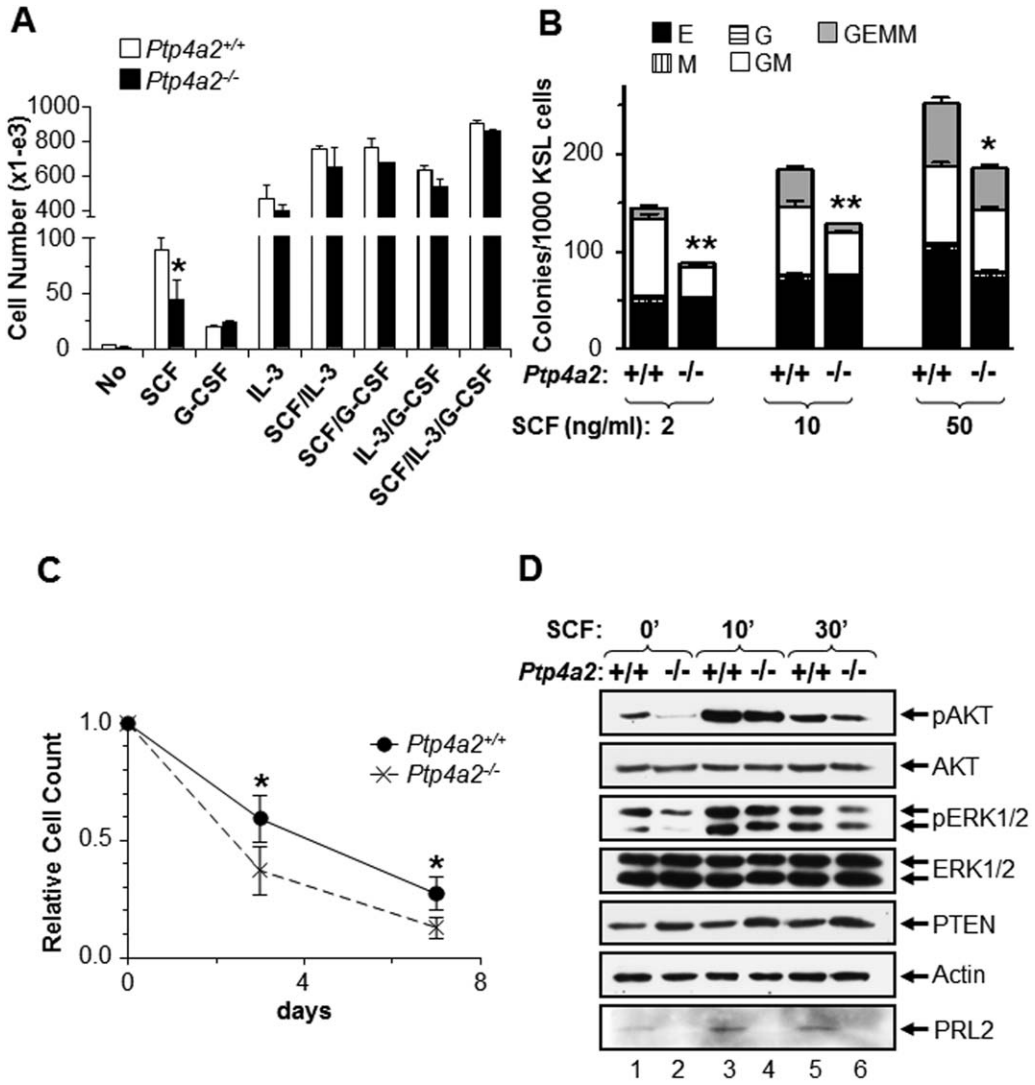

Figure 5. PTP4A2 is important for SCF/KIT signaling in hematopoietic stem and progenitor cells. **(A):** Proliferation of wild-type and *Ptp4a2* null Kit$^+$ cells in response to cytokine stimulation. Data shown are the mean values ± SD (*, $p < .05$, $n = 3$). **(B):** Colony-forming unit assays in the presence of increased concentration of SCF. Data shown are the mean values ± SD (**, $p < .01$, $n = 3$). **(C):** Proliferation of wild-type and *Ptp4a2* null Kit$^+$ cells in the absence of cytokines. Data shown are the mean values ± SD (*, $p < .05$, $n = 3$). **(D):** Immunoblot analysis of AKT and ERK phosphorylation in wild-type and *Ptp4a2* null Kit$^+$ cells. Data represent three independent experiments. Abbreviation: SCF, stem cell factor.

proteins implicated as biomarkers and therapeutic targets in cancer [23–25]. Individual PTP4As are overexpressed in a variety of cancer cell lines and tissues when compared with their normal counterparts [26–29]. However, the exact biological functions of PTP4As are largely unknown [23–25]. Herein we report that PTP4A2/PRL2 plays a critical role in HSC self-renewal and SCF/KIT signaling.

While loss of *Ptp4a2/Prl2* modestly increased the frequency of MPPs, *Ptp4a2* deficiency does not affect immunophenotypic HSC numbers. To further define the role of PTP4A2 in regulating HSC self-renewal, we performed serial bone marrow transplantation assays and found that loss of *Ptp4a2* impairs the ability of HSCs to repopulate the lethally irradiated recipient mice. These observations were confirmed by HSC transplantation. Furthermore, we observed no homing defects following transplantation and no increased HSC apoptosis, demonstrating that PTP4A2 functions as a positive regulator of HSC self-renewal. We also found that the percentage of donor-derived T cells was significantly lower in mice transplanted with *Ptp4a2* null cells than that with wild-type cells, indicating that PTP4A2 may also regulate T-cell differentiation under stress conditions.

Among the signal transduction pathways that have attracted considerable attention as possibly being involved in HSC maintenance is the phosphoinositide 3-kinase (PI3K)-AKT pathway [43]. As an antagonist of the PI3K pathway, PTEN has been implicated as a regulator of HSC self-renewal and loss of PTEN in the HSC compartment results in hyperproliferation of HSPCs [44]. Recently, we showed that in mouse placenta PTP4A2/PRL2 promotes cell proliferation by activating the AKT kinase through downregulation of the tumor suppressor PTEN [32]. It appears that *Ptp4a2* overexpression enhances the degradation of PTEN protein in these cells, indicating that PTP4A2/PRL2 regulates the stability of PTEN in placenta [32].

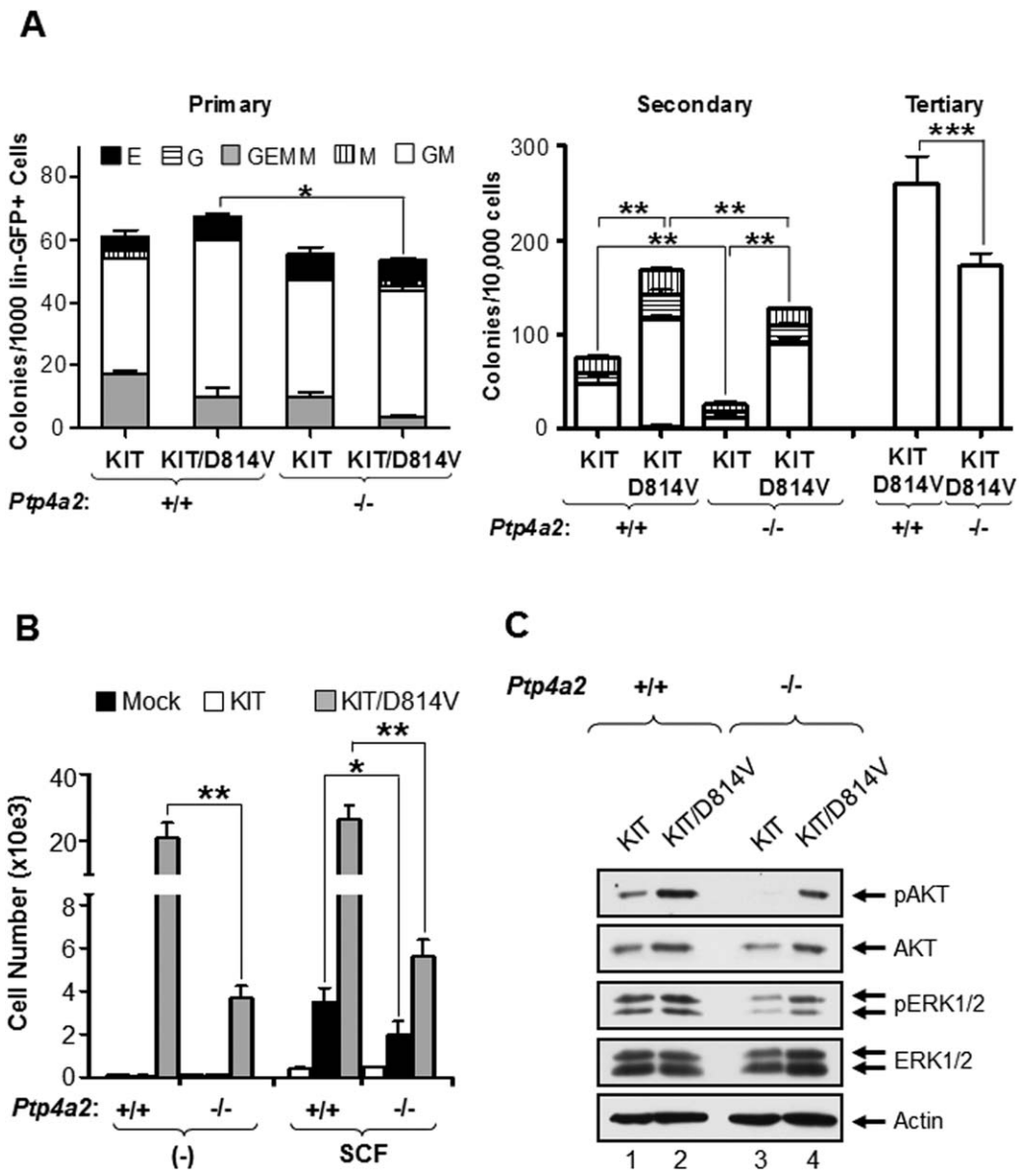

Figure 6. PTP4A2 is important for oncogenic KIT signaling in hematopoietic progenitor cells. **(A):** Myeloid progenitors were quantified by methylcellulose culture in the presence of SCF using wild-type and *Ptp4a2* null Lin^- cells transduced with retroviruses expressing the WT or the mutant form (KIT/D814V) of KIT. The methylcellulose cultures were serially replated, weekly, for 3 weeks. Data are means ± SD (*, $p < .05$; **, $p < .01$; ***, $p < .001$, $n = 3$ independent experiments). **(B):** Proliferation of wild-type and *Ptp4a2* null Lin^- cells expressing the WT or the mutant form (KIT/D814V) of KIT in the presence or the absence of cytokine stimulation. Data are means ± SD (**, $p < .01$, $n = 3$ independent experiments). **(C):** Immunoblot analysis of AKT and ERK phosphorylation in wild-type and *Ptp4a2* null Lin^- cells expressing the WT or the mutant form (KIT/D814V) of KIT in response to SCF stimulation. Data represent three independent experiments. Abbreviation: SCF, stem cell factor.

In addition, we observed that loss of *Ptp4a2* decreases HSPC proliferation and *Ptp4a2* null hematopoietic progenitor cells show decreased phosphorylation of AKT and ERK1/2 in steady state. To investigate how PTP4A2 regulates PI3K/AKT signaling in hematopoietic progenitor cells, we examined the level of PTEN in *Ptp4a2* null HSPCs. As seen in placenta, we observed moderate but statistically significant elevation of PTEN protein in *Ptp4a2* null HSPCs compared to that in wild-type HSPCs, whereas PTEN RNA level is identical between *Ptp4a2* wild-type and null cells. Therefore, it is possible that PTP4A2 regulates the stability and turnover of PTEN protein in HSPCs as well. PTEN has been shown to regulate cell proliferation and cancer progression in a dosage-dependent manner and a slight reduction in PTEN levels dictates cancer susceptibility [45, 46]. Thus, it is likely that increased level of PTEN we observed contributes to the decreased activation of AKT and ERK1/2 signaling pathways in *Ptp4a2* null HSPCs. Moreover, consistent with PTEN's established role in sustaining HSPC proliferation, our data suggest that increased PTEN expression in *Ptp4a2* deficient mice leads to reduced proliferation of HSPCs.

Ectopic expression of *Ptp4a2* in nontumorigenic cells has been shown to enhance cell cycle progression and promotes proliferation [28, 36]. This process may be dependent on the

downregulation of cyclin-dependent kinase inhibitor p21 [28]. The cell cycle defects seen in *Ptp4a2* null mice may also be mediated by cell cycle regulators as we observed increased expression of p21 and p57 in HSPCs. Both p21 [47] and p57 [48] have been shown to regulate HSPC proliferation; therefore, PTP4A2 plays an important role in promoting HSPC proliferation, at least in part by controlling the level of cell cycle regulators. How PTP4A2 regulates the expression of these cell cycle regulators is not clear. However, we note that PTEN controls cell cycle entry and progression through inhibiting PI3K-AKT activity [49, 50] and PTEN loss leads to enhanced G0-G1 cell cycle transition, which yields a ST expansion of HSCs [43].

Given that *Ptp4a2* null hematopoietic progenitor cells show decreased colony formation in serial replating assays, it is possible that PTP4A2 regulates cytokine signaling in hematopoietic progenitor cells. While *Ptp4a2* null hematopoietic progenitor cells proliferate normally in response to IL-3 or G-CSF stimulation, these cells are less proliferative in response to SCF stimulation. Furthermore, *Ptp4a2* null hematopoietic progenitor cells show decreased colony formation in response to increased concentrations of SCF compared with wild-type cells. The role of PTP4A2 in mediating SCF/KIT signaling appears modest, as SCF still activates a remarkable amount of AKT and ERK in *Ptp4a2* null cells (Fig. 5D). Therefore, PTP4A2 may be one of many factors that contribute to KIT function in HSPCs.

As the receptor for SCF, the biologic significance of KIT in hematopoiesis was first revealed in the white spotting (*W*) mutant mice [51]. Several *W* mutations with different levels of KIT kinase deficiency were found to correlate with the phenotype severity [9–14]. Studies in the viable primary *W* mutant mice or in mice after transplantation indicate the importance of KIT signaling in maintaining HSC quiescence and survival [9–14]. The hematopoietic defects seen in the *Ptp4a2* null mice recapitulate some hematopoietic phenotype of KIT mutant mice, providing additional evidence that PTP4A2 plays an important role in mediating SCF/KIT signaling in hematopoietic progenitor cells.

While *Ptp4a2* is highly expressed in acute myeloid leukemia cells [35, 36], its role in leukemogenesis is largely unknown. We discovered that loss of *Ptp4a2* decreased the ability of KIT/D814V mutant in promoting hematopoietic progenitor cell proliferation, demonstrating that PTP4A2 is an important effector molecule of oncogenic KIT signaling in HSPCs and positively contributes to KIT/D814V-induced hematopoietic progenitor hyperproliferation. Therefore, pharmacological inhibition of PTP4A2 in KIT/D814V-expressing cells in vivo could delay leukemia progression and decrease the severity of the disease.

Conclusions

In summary, this study reveals a critical role of PTP4A2/PRL2 in HSC self-renewal and SCF/KIT signaling. Our results suggest that the PTP4A2 phosphatase may be a druggable target in MPD and AML with oncogenic KIT mutations.

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Author Contributions

M.K.: designed the research, performed the research, analyzed the data, performed the statistical analysis, and wrote the manuscript; R.K., M.C.Y., and Z.-Y.Z.: designed the research and wrote the manuscript; Y.L.: designed the research, analyzed the data, performed the statistical analysis, and wrote the manuscript; Y.D. and H.Y.: performed the research, analyzed the data, and performed the statistical analysis; Y.B., S.C., and L.Z.: performed the research.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Functional B-1 progenitor cells are present in the hematopoietic stem cell-deficient embryo and depend on *Cbfβ* for their development

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The fetal liver is a major hematopoietic site containing progenitor cells that give rise to nearly all blood cells, including B-1 cells. Because the fetal liver is not a de novo site of hematopoietic stem cell (HSC) or progenitor-cell emergence, it must be seeded by yolk sac (YS)-derived erythromyeloid progenitors at embryonic day (E) 8.5–E10 and aorta-gonado-mesonephros (AGM)-derived HSCs at E10.5–E11.5. Although the B-1 progenitor cell pool in the fetal liver is considered to be of HSC origin, we have previously proposed that YS-derived B-1 progenitors may also contribute to this pool. Until now, it has been impossible to determine whether HSC-independent B-1 progenitor cells exist in the fetal liver. Here, we demonstrate the presence of transplantable fetal-liver B-1 and marginal zone B progenitor cells in genetically engineered HSC-deficient embryos. HSC-deficient YS and AGM tissues produce B-1 progenitors in vitro and thus may serve as sites of origin for the B-1 progenitors that seed the fetal liver. Furthermore, we have found that core-binding factor beta (*Cbfβ*) expression is required for fetal-liver B-1 progenitor cell maturation and expansion. Our data provide, to our knowledge, the first evidence for the presence of B-1 progenitor cells in the fetal liver that arise independently of HSCs and implicate *Cbfβ* as a critical molecule in the development of this lineage.

TEK | hemogenic endothelial cell | immune layered model

The immune layered model proposed by Herzenberg and Herzenberg in 1989 (1) predicted B-1 and B-2 lineage production through three waves of B lymphopoiesis arising from different precursors during development (2): According to this model, the first wave gives rise exclusively to innate immune B cells in early embryonic life and may be derived from progenitor cells independent of hematopoietic stem cells (HSCs), challenging the stem-cell theory that all blood cells are products of HSCs. The second and third waves are comprised of HSCs and HSC-derived progenitors in the fetal liver, neonatal bone marrow (BM) (the second wave), and adult BM (the third wave), respectively. Importantly, $AA4.1^{+}CD19^{+}B220^{lo\text{-}neg}$ B-1 specific progenitors have been identified in the second wave (3). The second wave produces more B-1 cells than B-2 cells whereas the third wave displays an opposite skewing of B-cell differentiation (4–7). In fact, the B-1 cell-producing abilities of HSCs and common lymphoid progenitor cells decline with advancing age (6), and, in particular, $CD5^{+}$B-1a cells are not produced by adult HSCs when examined by single HSC transplantation assay (7). Although the second and third waves have been examined in detail, it is unclear whether the first wave exists and contributes to innate immunity in postnatal life and whether the B-1 progenitor cells in wave 2 in the fetal liver are all HSC-derived or contain derivatives of the wave 1 HSC-independent embryonic progenitor cells.

Murine B-1 cells are innate immune cells (distinguished from conventional B-2 cells by specific surface markers such as $IgM^{hi}IgD^{lo}CD11b^{+}$), residing in the peritoneal and pleural cavities. These cells produce stereotypic natural antibodies in a T cell-independent manner and execute important roles in the first line of defense against microbial infection (8, 9). B-1 cells are segregated into $CD5^{+}$B-1a and $CD5^{-}$B-1b cells. Marginal zone (MZ) B cells, named after the restricted localization of these cells in the splenic marginal zone, are usually categorized as BM HSC-derived B-2 cells but share similar functions with B-1 cells, such as rapid production of IgM antibodies against bacterial pathogens in a T cell-independent manner. There is evidence that a portion of MZ B cells is also of embryonic or fetal origin (10–12).

We have recently reported that yolk sac (YS) and para-aortic-splanchnopleura (P-Sp) hemogenic endothelial cells (HECs) harvested before the first emergence of HSC give rise to transplantable, functional B-1a, B-1b, and MZ B cells in vitro and thus have provided supportive evidence for the first wave of B cells (13). However, because we isolated and cultured YS/P-Sp cells in vitro to allow them to differentiate into B-1 progenitor cells, whether YS/P-Sp–derived B progenitor cells seed the fetal liver in vivo and contribute to the B-1 progenitor cell pool or mature B-1 or MZ B cells in postnatal life has never been established. In other words, to address the question whether the first wave of B lymphopoiesis is present in vivo or not, we have to confirm the existence of HSC-independent B-1 progenitor cells in the fetal liver.

The fetal liver is an organ dependent upon hematopoietic stem/progenitor cell seeding from different hematopoietic tissues.

Significance

All lymphoid cells are considered to be products of hematopoietic stem cells (HSCs); however, it has been suggested, but not proven, that innate immune B-1 progenitor cells develop independently of HSCs in the fetal liver. B-1 cells, especially B-1a cells, are not replaced by adult bone marrow transplantation. Thus, it is critical to understand the origin and mechanisms required to sustain these cells in vivo because B-1 cells play important roles in the first line of defense against microbial infection and in preventing organ damage in autoimmune patients and infections in some patients after bone-marrow transplantation. We demonstrate that B-1 progenitor cells can develop independently of HSCs in the fetal liver and that their development relies critically on the expression of core-binding factor beta.

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It is an established concept that erythro-myeloid progenitors (EMPs) derived from embryonic day (E) 8.5–E10 YSs seed the fetal liver to support homeostatic hematopoiesis in the embryo whereas HSCs that emerge in the aorta-gonado-mesonephros (AGM) region seed the fetal liver at E11 and provide hematopoietic support later in development (14, 15). However, it is unknown whether the YS/P-Sp HEC-derived B-1 lymphoid progenitors seed the fetal liver. Because the B-1 progenitor cell pool in the fetal liver is considered to be an HSC derivative and because HSCs exist in the fetal liver concomitant with B-1 progenitor cells, it has been impossible to prove the existence of HSC-independent B lymphopoiesis in the fetal liver. To specifically address this question, we have used a unique mouse model devoid of HSC but known to possess some uncharacterized fetal-liver B cells (16, 17).

Core-binding factor beta (CBFβ) is the common non–DNA-binding subunit of the *Cbf* family of heterodimeric transcription factors. By associating with CBFα subunits, CBFβ increases the affinity of CBFα DNA-binding. Runt related transcription factor 1 (*Runx1*) also called core-binding factor alpha 2 (*Cbfα2*)$^{-/-}$ embryos die at E12 and display severe deficiencies in definitive EMP, lymphoid cells, and HSCs (18). CBFβ is required for Runx1 function, and loss of CBFβ in embryos leads to embryonic lethality at E11–E13 similar to *Runx1*$^{-/-}$ embryos with a severe defect in definitive hematopoiesis and HSCs (17, 19). However, when *Cbfβ* expression was restored in *Cbfβ*$^{-/-}$ embryos under transgenic control of the endothelial promoter endothelial-specific receptor tyrosine kinase (Tek) (*Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ*), EMP formation and rare B and T cells were observed (16). In these rescued embryos, lymphoid progenitors (Lin$^-$c-Kit$^+$Flt3$^+$) were detectable in the E14 fetal liver and produced B or T cells in OP9 or OP9-DL1 in vitro cocultures, respectively. However, E14 *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal-liver cells failed to reconstitute recipient peripheral blood (PB) and BM upon transplantation, indicating that *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos contain lymphoid progenitors without transplantable HSCs. It has been previously reported that lymphoid progenitor cells are present in the YS and P-Sp before HSC emergence at an early stage (E8.25–E9.5) of embryo development (13, 20–23). Furthermore, the presence of multipotent progenitor cells with myeloid, B-cell, and T-cell lineage potentials without long-term HSC activity also has been reported (24–26). Whether the B and T progenitor cells emerge from a common HEC or are independently generated has not been fully resolved. Nonetheless, the fact that lymphoid progenitor cells are present prior to HSC emergence suggests that the initial phases of developmental hematopoiesis may not follow the same paradigm as adult BM HSC development where all of the lymphoid progenitor cells are HSC-derived (15, 27). Indeed, transplantable HSCs were rescued when *Cbfβ* was expressed under transgenic control of the *Ly6a* promoter in the *Cbfβ*$^{-/-}$ embryos (*Cbfβ*$^{-/-}$*:Ly6a-GFP/Cbfβ*), suggesting that *Cbfβ* is required in the Ly6a$^+$ HECs for producing transplantable HSCs that repopulate myeloid and lymphoid lineages (16). Based on these findings and our previous reports that B-1 (but not B-2) and T lymphoid progenitor cells are present in the embryo before HSC emergence (13, 20), we hypothesized that the unspecified B cells detected in the *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos that do not harbor transplantable HSCs are HSC-independent B-1 progenitor cells that constitute the first wave of B lymphopoiesis.

In the present study, we confirm the existence of fetal-liver AA4.1$^+$CD19$^+$B220$^-$ B-1 progenitor cells in the absence of transplantable HSCs in vivo. YS and AGM cells from *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos produce B-1 progenitor cells in vitro. Our results clearly demonstrate the presence of B-1 and MZ B progenitor cells in the fetal liver independent of HSCs and suggest that these progenitor cells are likely to be derived in part from YS/P-Sp sites. However, these B-1 progenitor cells do not display persistent expression of *GFP/Cbfβ* because *TEK* is not expressed once HECs become hematopoietic cells. Therefore, this *Cbfβ* deficiency in B-1 progenitor population results in impaired maturation and expansion of these cells. Consistently, we show that overexpression of *Cbfβ* in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal-liver cells via a retrovirus promotes engraftment of B-1 and MZ B cells in recipient immunodeficient mice. Thus, our findings demonstrate that persistent *Cbfβ* expression is required for the maturation and expansion of B-1 progenitor cells during in vivo development.

Results

B-1 Progenitor Cells Exist in the Fetal Liver in the Absence of HSCs. First, we examined whether AA4.1$^+$CD19$^+$B220$^{lo\text{-}neg}$ B-1 progenitor cells exist in the fetal liver of *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos. As previously reported (16), *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos can survive until birth in the absence of HSCs. The fetal livers of *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos at E15.5 and E17.5 were of similar size to the fetal liver of wild-type (WT) littermate embryos. Total fetal-liver mononuclear cell (MNC) counts from E17.5 *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos were significantly lower than WT embryos (Fig. 1*B*). As shown in Fig. 1*A*, lin$^-$AA4.1$^+$CD19$^+$B220$^{lo\text{-}neg}$ B-1 progenitor cells were detected in the fetal livers of *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos from E15.5 until birth although the frequency and total number were significantly lower than WT values (Fig. 1 *A–D* and *M*). Furthermore, lin$^-$AA4.1$^+$CD19$^+$B220$^{lo\text{-}neg}$ B-1 progenitor cells were also detected in the fetal spleen from E15.5 until birth (Fig. 1 *G–J* and *N*). Few AA4.1$^+$CD19$^+$B220$^+$ B cells were detected in the fetal liver and spleen of *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos whereas WT fetal liver contained more AA4.1$^+$CD19$^+$B220$^+$ B cells than B-1 progenitor cells (Fig. 1 *A*, *E–G*, *K*, and *L*). AA4.1$^+$CD19$^+$B220$^+$ cells are overlapping with AA4.1$^+$B220$^+$CD43$^+$ proB cells (5). Whereas adult BM AA4.1$^+$CD19$^+$B220$^+$ cells give rise predominantly to B-2 cells, fetal-liver AA4.1$^+$CD19$^+$B220$^+$ or AA4.1$^+$B220$^+$CD43$^+$ cells are known to give rise mainly to B-1 cells upon transplantation (3, 5). Thus, the fetal-liver AA4.1$^+$CD19$^+$B220$^+$ population contains more B-1 lineage than B-2 lineage cells and is considered to be differentiated from both HSCs (through AA4.1$^+$CD19$^-$B220$^+$ phenotype) and B-1 progenitor cells as we have previously reported (13). Therefore, severe reduction of AA4.1$^+$CD19$^+$B220$^+$ cells in the fetal liver (Fig. 1 *E* and *F*) and spleen (Fig. 1 *K* and *L*) is due to HSC deficiency and an impaired maturation of HSC-independent B-1 progenitor cells in the *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos. Nevertheless, it is of note that *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal spleen contains B progenitor cells ($2.0 \times 10^4 \pm 1.7 \times 10^4$ cells) at 44% of the number measured in WT fetal spleen ($4.7 \times 10^4 \pm 1.3 \times 10^4$ cells) (by calculating total B progenitor cells in E17.5 spleen) (Fig. 1*N*). This result suggests that at least 40% of B-1 progenitor cells in the fetal-neonatal spleen are HSC-independent, a point that contrasts with the common perception that all lymphoid cells are HSC-derived.

B-1 Progenitors in Cbfβ$^{-/-}$:Tek-GFP/Cbfβ Fetal Liver Fail to Mature and Expand in Vitro. Although we have confirmed for the first time, to our knowledge, that B-1 progenitors are present in the HSC-deficient fetal liver, we have also observed a possible maturation defect of B-1 progenitor cells in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos (Fig. 1). To examine the maturation capacity of B-1 progenitor cells in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal liver, AA4.1$^+$CD19$^+$B220$^{lo\text{-}neg}$ B-1 progenitor cells from WT and *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal liver were plated on OP9 stromal cells with added IL-7 and Flt3-ligand. WT AA4.1$^+$CD19$^+$B220$^{lo\text{-}neg}$ cells proliferated, forming cobblestones beneath the stromal cells, and produced more than 10^6 nonadherent cells with CD19$^+$B220$^+$ (including IgM$^+$) B cells in 7–10 d (Fig. 2*A*, *Upper*). In contrast, *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* AA4.1$^+$CD19$^+$B220$^{lo\text{-}neg}$ cells did not proliferate as robustly although they produced cobble stones by

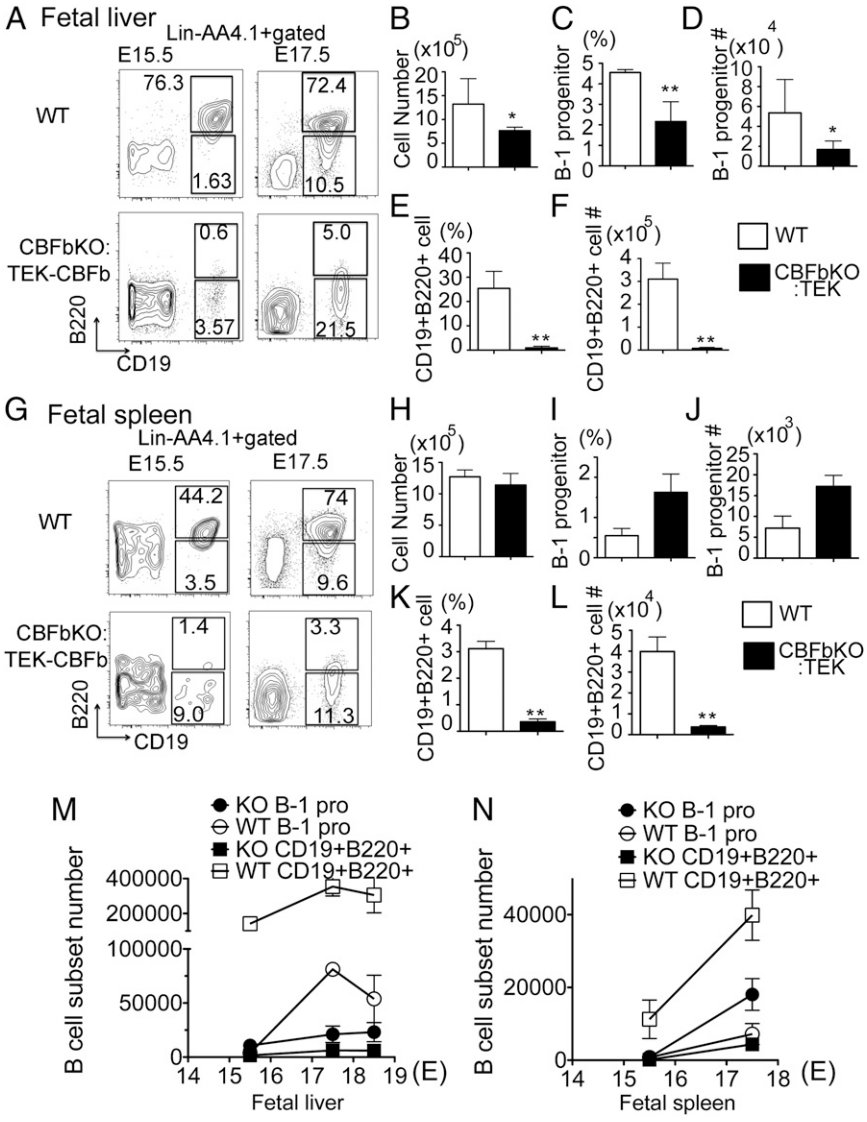

Fig. 1. B-1 progenitor cells exist in the *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal liver (FL) and spleen. AA4.1^{+}CD19^{+} B220$^{lo\text{-}neg}$ B-1 progenitor phenotype was examined in the WT and *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* fetal-liver and spleen cells at each day from E15.5 until birth (E18.5). The representative data for E15.5 and E17.5 are depicted. Lin^{-}AA4.1^{+} gated FACS dot plots at E15.5 and E17.5 fetal liver (*A*) and fetal spleen (*G*) are depicted. Total MNCs of E17.5 FL (*B*) and spleen (*H*), percentage of B-1 progenitor cells in FL (*C*) and spleen (*I*), total cell number of B-1 progenitor cells in FL (*D*) and spleen (*J*), percentage of AA4.1^{+}CD19^{+} B220^{+} cells in FL (*E*) and spleen (*K*), and total cell number of AA4.1^{+}CD19^{+}B220^{+} cells in FL (*F*) and spleen (*L*) are shown. (*B–F* and *H–L*) Open bar, WT; filled bar, *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ*. The cell numbers of B-1 progenitors and CD19^{+}B220^{+} cells from WT and *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* FL (*M*) and spleen (*N*) at each embryonic day are shown (n = 4 for each group; *<0.05, **<0.01).

17 d with 10^6 nonadherent cells, including a few CD19^{+}B220^{+} cells and many Mac1^{+} macrophages (Fig. 2*A*, *Lower*). Considering the fact that AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ cells were originally reported as B cell-macrophage bipotent progenitors (28), these results indicated that loss of *Cbfβ* may have contributed to the reduction of B lymphoid potential in this population. This is compatible with a previous report that the B lymphoid potential of lymphoid progenitor cells in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos was 30-fold less than in WT embryos (16). Thus, proliferation and B-1 cell differentiation capacity were significantly impaired in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* B-1 progenitor cells at least under the coculture conditions we used in vitro.

YS and AGM Can Produce B-1 Progenitor Cells in the Embryo Devoid of HSCs. The fetal liver is not a de novo site of hematopoietic cell emergence and must be seeded by various hematopoietic progenitor/stem cells derived from other hematopoietic tissues (15). Because HSCs are not produced in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos, we examined the possible source of B-1 progenitor cells that seed the fetal liver. We cocultured YS and P-Sp/AGM cells with OP9 stromal cells as previously reported (13). As expected, *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* YS and AGM cells produced AA4.1^{+} CD19^{+}B220^{+} B cells but at a lower frequency compared with WT YS/AGM (Fig. 2*B*). These cultured YS/P-Sp/AGM-derived AA4.1^{+}CD19^{+}B220^{+} B progenitor cells were confirmed to become mature B-1 cells in vivo upon transplantation as previously reported (13). Although there is a study suggesting that placenta has the potential of producing B cells at a pre-HSC stage (29), we found maternal B cells in the placenta at this time point whereas YS and P-Sp derived B-1 cells were confirmed to be embryonic in origin (13). Thus, although we cannot exclude the possibility of the placenta as a production site for the first wave of B-1 progenitor cells in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos, our data indicate that YS and AGM produce B-1 progenitor cells that may seed the fetal liver in the *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos devoid of HSCs.

B-1 Progenitor Cells Can Mature into B-1 and MZ B Cells in Vivo but at Low Efficiency. Lin^{-}AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ B-1 progenitor cells were maintained until birth in *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* mice (B progenitor cells were present at 40% of the number of WT mice in the spleen) (Fig. 1*N*), and mice died 1 d after birth as previously reported (16). Because mature B-1 cells are not detectable in the peritoneal cavity until 5 d after birth (30), we were unable to find mature peritoneal B-1 cells in the *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* live-born neonates. To examine whether the B-1 progenitor cells found in the fetal liver of *Cbfβ*$^{-/-}$*:Tek-GFP/Cbfβ* embryos can mature into B-1 cells in vivo, we transplanted fetal-liver MNCs

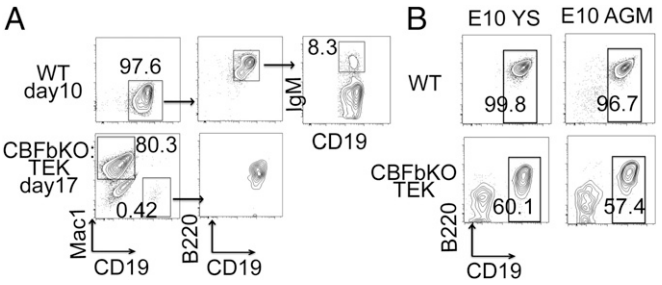

Fig. 2. (*A*) B-1 progenitor cells from *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal liver fail to expand in vitro. AA4.1^{+}CD19^{+}B220$^{lo\text{-}neg}$ B-1 progenitor cells were sorted from E17.5 WT and *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* FL and plated on OP9 stromal cells with IL-7 and Flt3-ligand. FACS dot plots of WT culture on day 10 (*Upper*) and *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* culture on day 17 (*Lower*) are depicted. Representative FACS dot plots from three separate experiments are depicted. (*B*) YS and AGM cells from *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* embryos can produce B progenitor cells in vitro. E10 YS and AGM from WT (*Upper*) and *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* (*Lower*) embryos were plated on OP9 stromal cells with IL-7 and Flt3-ligand. Cells after 10-d coculture were analyzed for their surface markers by flow cytometry. Representative FACS dot plots from two separate experiments (each experiment was done in triplicate) are depicted.

from WT and *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* embryos into irradiated NOD/SCID/IL2γc$^{-/-}$ (NSG) neonates. Although WT fetal-liver MNCs displayed multilineage repopulation in the PB of the recipient mice (Fig. 3*A*, *Upper*), no apparent engraftment was found in the PB of the recipient mice transplanted with *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver MNCs as previously reported (16) (Fig. 3*A*, *Lower*). However, in the host peritoneal cavity and spleen, B-1 and MZ B cells were reconstituted from donor *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver MNCs (Fig. 3*B*). No donor-derived B-2 cells or T cells were detected (Figs. 3*B* and 4*E*). These data indicated that HSC-deficient fetal-liver MNCs contain progenitor cells that can mature into only B-1 and MZ B cells in vivo upon transplantation.

CBFβ Is Required for Maturation and Expansion of B-1 and MZ B Cells. Although transplantable B-1 and MZ B-cell progenitors are present in HSC-deficient fetal liver, the level of chimerism was significantly lower than donor contributions from WT embryos (WT 97.8 ± 1.3% vs. *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* 1.98 ± 0.3% in the peritoneal cavity and WT 92.8 ± 3.3% vs. *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* 3.4 ± 2.4% in the spleen). It has been reported that *Cbfβ* is required in all blood-lineage cells for their development, but *TEK-GFP/Cbfβ* expression was not sustained once *Tek-GFP/Cbfβ*$^{+}$ HECs become hematopoietic cells (16, 17). We confirmed that WT fetal-liver B-1 progenitor cells express *Cbfβ* at a similar level to HSCs (Fig. 4*A*). However, B-1 progenitor cells from *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* do not express *TEK-GFP/Cbfβ* (Fig. 4*B*), thus having lost *Cbfβ* expression during differentiation from *TEK*$^{+}$ HEC to B-1 progenitor cells (Fig. 4*C*). We have also recently determined that loss of *Runx1* or *Cbfβ* in the B-cell lineage caused B-2 cell maturation defects (31). In this mouse model of *Cbfβ* deficient in B-cell lineage, we also observed a threefold reduction in B-1 cell number compared with WT mice, indicating that *Cbfβ* plays a critical role in B-1 cell development (Fig. S1) (31). Thus, we hypothesized that *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver B-1 progenitor cells would require ongoing *Cbfβ* expression to mature into functional B-1 cells. To test the hypothesis, we overexpressed *Cbfβ* in *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver MNCs using a retrovirus vector that constitutively produced CBFβ (Fig. 4*D* and Fig. S2*A*). Once retrovirally infected fetal-liver *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* MNCs were transplanted into irradiated NSG neonates, *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ*-derived cells displayed dramatically improved engraftment capacity in up to 49% of donor cells in the peritoneal cavity (Fig. 4*E*) and improved maturation into IgM^{+} cells in the spleen (Fig. S3), and displayed only B-1 cell reconstitution in the peritoneal cavity and MZ and B-1 cells in the spleen 12 wk after transplantation (Fig. 4 *E* and *F* and Fig. S3). Nearly all (98%) of these *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ*-derived cells expressed retroviral Cbfβ-GFP (Fig. S2*B*) and displayed 15–25 times more *Cbfβ* mRNA expression compared with WT peritoneal B-1 cells (Fig. S2*C*). In contrast, *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells infected with the empty vector control virus poorly engrafted (Fig. 4*E*, *Middle*). These results indicate that *Cbfβ* overexpression rescues maturation and expansion of impaired B-1 progenitor cells from *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* embryos. Furthermore, *Cbfβ* expressing engrafted B-1 cells in the recipient mice were functional, displaying T cell-independent antiphosphorylcholine IgM secretion detected by enzyme-linked immunospot (Elispot) assay (Fig. 4 *G* and *H*). Taken together, *Cbfβ* reexpression restored engraftment and expansion/maintenance capacity of HSC-independent B-1 and MZ B cells from *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal liver in vivo, suggesting that *Cbfβ* plays important roles in B-1 cell development and proliferation, similar to B-2 cell development (31). Of note, HSCs were not restored by retrovirus *Cbfβ* overexpression in *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells: no engraftment of any hematopoietic lineages other than B-1 or MZ B cells (Fig. 4*E*, *Bottom*, Fig. 4*F*, and Fig. S3), suggesting that the timing and dose of *Cbfβ* expression may be differentially required for the development of each hematopoietic lineage.

Discussion

In the current study, using unique HSC-deficient embryos, we have for the first time, to our knowledge, proven that progenitor cells that give rise to only B-1 and MZ cells exist in the fetal liver in the absence of HSCs. Although we were not able to quantitatively estimate the percentage contribution of the first wave of B cells in postnatal life (because the *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* neonates die just after birth), it is probable that at least 40% of

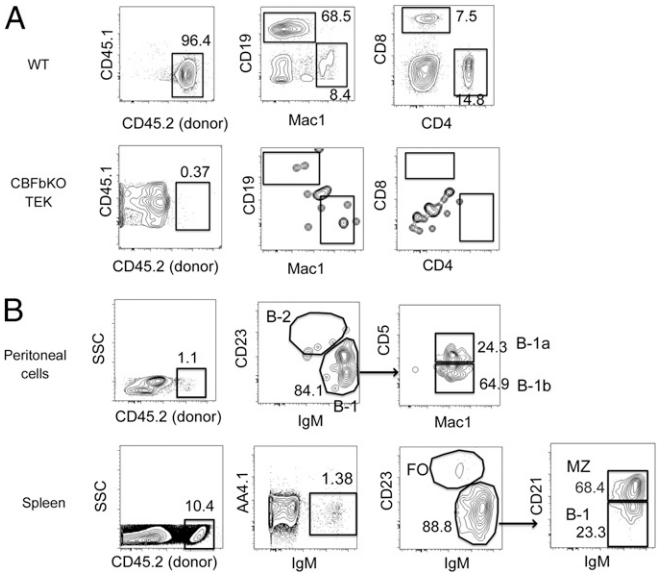

Fig. 3. *Cbfβ*$^{-/-}$:*Tek-GFP/CBFβ* fetal-liver cells contain a few B-1 and MZ progenitor cells, but not HSCs. E15.5 FL MNCS from WT and *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* embryos were transplanted into sublethally irradiated NSG neonates. PB analysis 8 wk after injection is depicted (*A*). PB of recipient mice transplanted with WT (*Upper*) and *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* (*Lower*) fetal-liver cells are depicted. (*B*) The peritoneal (*Upper*) and spleen (*Lower*) cells in the recipient mice transplanted with *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells are shown. FO, follicular B cell.

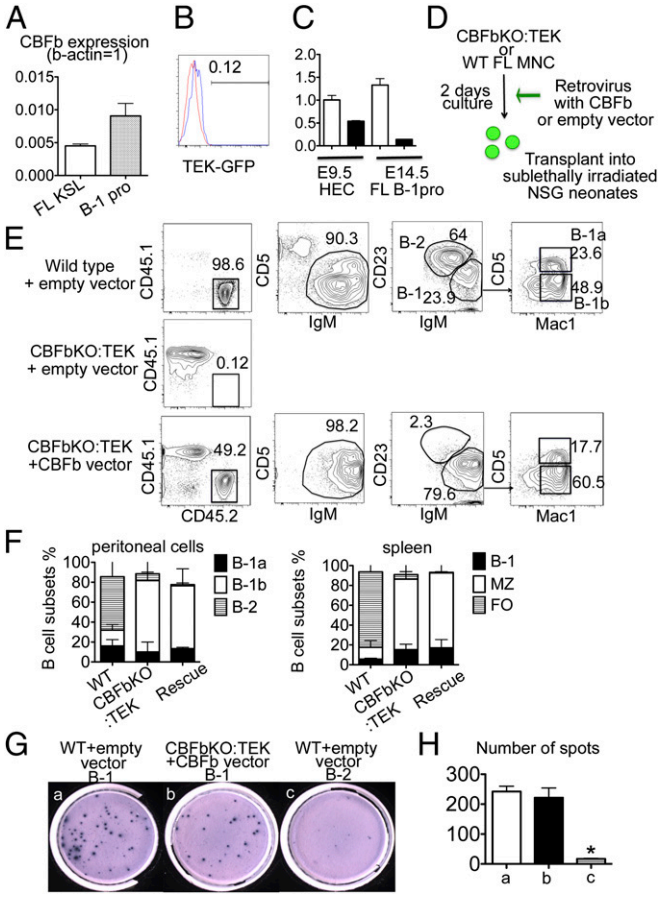

Fig. 4. Overexpression of *Cbfβ* in *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells restored B-1 and MZ cell engraftment. (*A*) *Cbfβ* expression in WT fetal-liver HSCs and B-1 progenitors by quantitative PCR is shown. Internal control: β-actin = 1.0. (*B*) *Tek-GFP/Cbfβ* expression within E15.5 fetal-liver AA4.1^{+} CD19^{+}B220^{dim} B-1 progenitor cells in *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* embryos. Red line, WT; blue line, *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver B-1 progenitor cells. (*C*) Relative *Cbfβ* expression in E9.5 WT CD31^{+}Tie2^{+} HECs, E9.5 *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* HECs, E14.5 WT FL B-1progenitors, and E14.5 *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* FL B-1 progenitors, compared with E9.5 WT HECs (=1). Open bar, WT; filled bar, *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ*. (*D*) Experimental design for rescuing *Cbfβ* expression in *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells. (*E*) Peritoneal cells of recipient mice at 12 wk after transplantation with fetal-liver cells from WT (*Top*) or *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells expressing empty vector (*Middle*) or *Cbfβ* (*Bottom*), respectively. (*F*) Donor-derived B-cell subsets in the recipient peritoneal cavity (*Left*) and spleen (*Right*) are depicted. Donor cells were WT fetal-liver MNC cells (left bars), *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells (center bars), and *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells infected with *Cbfβ* expressing retrovirus (rescue, right bars), in each panel. (*G*) IgM secretions from B-1 or B-2 cells stimulated with phosphorylcholine were detected by Elispot assay: (*a*) 1,000 B-1 cells from the recipient mice transplanted with WT fetal-liver cells with empty vector, (*b*) 650 B-1 cells derived from *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal-liver cells with *Cbfβ* expression vector, and (*c*) 1,000 B-2 cells derived from WT fetal-liver cells with empty vector were initially plated. (*H*) The numbers of spots per 1,000 B-1 or B-2 cells derived from recipient mice in Elispot assay (*G*, *a*, *b*, and *c*) are depicted. There was no significant difference between B-1 cells from WT fetal liver with empty vector and from *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal liver with *Cbfβ* expression vector. The number of IgM-secreting B-2 cells was significantly less than that of B-1 cells from WT or *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* with *Cbfβ* expression vector (<0.05).

neonatal spleen B cells are derived from HSC-independent progenitors. Since fetal-liver cells do not produce any blood cells de novo but rely upon seeding from other sites, these B-1 progenitor cells must have been seeded from another site(s) of embryonic hematopoiesis, such as the yolk sac or embryo proper.

We also report that progenitor cells for MZ B cells developed in the absence of HSCs in the *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* embryos. We previously identified YS/P-Sp–derived B-1 progenitor cells as giving rise to MZ B cells upon transplantation in vivo (13). The *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* mouse model was instrumental in again validating the HSC-independent origin of MZ B cells. Because MZ B cells are also produced by adult BM HSCs and splenic lin^{-}CD19^{+}B220$^{lo\text{-}neg}$CD43^{-} precursors (7, 32), we speculate that MZ B cells may comprise precursors from heterogenous sites. Ito et al. have reported less frequent generation of MZ B than B-1 cells from YS lymphoid progenitors (33) but still showed some MZ B-cell potential of the YS precursors. Differences in their results from our present work may be the result of differences in coculture lines and choice of cytokines or cytokine concentrations. Ectopic expression of Lin28b has been reported to reprogram adult hematopoietic stem/progenitor cells into cells with the ability to give rise to fetal lymphopoiesis (34). In this report, Lin28-expressing cells differentiated into predominantly B-1a and MZ B cells in the recipient mice, in agreement with our results. Thus, accumulating evidence supports a paradigm that a part of MZ B cells are derived from embryonic precursors, similar to B-1 cells.

Another important finding from our current work is that *Cbfβ* expression is required for B-1 cell development as well as B-2 cell development as previously reported (31). However, B-1 cell maturation is permissive in *Cbfβ*$^{-/-}$:*Tek-GFP/Cbfβ* fetal liver-derived B-1 progenitor cells, and the reduction of mature B-1a cells in *Cbfβ*$^{flox/flox}$:*MB1-cre* mice is milder than the B-2 lineage. *Cbfβ* may play different roles in the B-1 and the B-2 cell lineages, and further study would be required to fully explore the roles played in each lineage.

In sum, the presence of transplantable B-1 and MZ progenitor cells in the fetal liver devoid of HSCs confirms the first wave of B lymphopoiesis in the immune layered model predicted more than 20 y ago (1). Many questions remain to be addressed. How do B-1 and MZ B-cell progenitors migrate into the fetal liver, spleen, and BM? How and where do the B-1 progenitor cells mature? How much do they really contribute to innate immunity compared with fetal-liver HSC-derived B-1 and MZ B cells? Lineage-tracing models would be required to resolve these questions, and answering those questions would help to understand the roles of B-1 cells in autoimmunity (35, 36), prevention of atherosclerosis (37), and prevention of infections in blood stem cell transplanted patients (38).

Materials and Methods

Mice. *Cbfβ*$^{+/-}$: *Tek-GFP/Cbfβ* mice were generated by crossing *Cbfβ*$^{+/-}$ mice and *Tek-GFP/Cbfβ* mice (kindly provided by Dr. Nancy Speck, University of Pennsylvania, Philadelphia). To obtain *Cbfβ*$^{-/-}$: *Tek-GFP/Cbfβ* embryos, timed mating was performed by crossing *Cbfβ*$^{+/-}$: *Tek-GFP/Cbfβ* males with *Cbfβ*$^{+/-}$: *Tek-GFP/Cbfβ or Cbfβ*$^{+/-}$ females. Embryonic tail was used for genotyping.

Transplantation. NSG neonates were used as recipients. One- to 5-d-old neonates were sublethally irradiated (150 rad), and fetal-liver cells from WT and *Cbfβ*$^{-/-}$: *Tek-GFP/Cbfβ* embryos were injected into the peritoneal cavity of NSG neonates. Six to 12 wk after injection, PB, peritoneal, and spleen cells were analyzed donor cell types in the recipient mice.

OP9 Culture. An OP9 stromal cell line was maintained with α-MEM and 20% (vol/vol) FBS. YS, P-Sp/AGM, or fetal-liver cells were plated on six-well plates confluent with OP9 stromal cells in induction medium [α-MEM, 10% (vol/vol) FBS and 5×10^{-5} M β-mercaptoethanol] supplemented with 10 ng/mL IL-7 and 10 ng/mL Flit3-ligand (PEPROTECH) as previously reported (13). Floating cells in the culture medium were collected at optimal time points, and we analyzed their surface markers by flow cytometry.

Flow Cytometry. For analysis and sorting of B-1 progenitors, B-1, MZ B Cells, and each lineage of blood cells, the following antibodies were used at different fluorescent color combinations: anti-mouse AA4.1 (AA4.1), CD19 (1D3), B220 (RA3-6B2), IgM (II/41), CD21 (8D9), CD23 (B3B4), CD11b (M1/70),

CD5 (53-7.3), CD3e (145-2C11), Ter119 (TER-119), c-kit (2B8), Sca-1 (D7), and IL-7Ra (A7R34) (all purchased from eBioscience). A lineage mixture containing anti-Ter119, CD11b, and CD3 was used for lineage negative gating. Cells were analyzed on LSRII or sorted on FACS Aria (Becton Dickinson).

Quantitative PCR. Fetal-liver $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ cells or $AA4.1^{+}CD19^{+}B220^{lo\text{-}neg}$ B-1 progenitors were sorted, and RNA was extracted using RNasy (QIAGEN) following the manufacturer's instructions. cDNA was made using Super Scrip III fast strand Synthesis System (Invitrogen). Real-time PCR was performed using FastStart Universal SYBR Green Master (Rox) (Roche) by 7500 Real-Time PCR System (Life Technologies). The following sets of primers were used: *Cbfβ*, forward, GCAAGTTCGAGAACGAGGAG; reverse, CCCGTGTACTTAATCTCGCA; *β-actin*, forward, TCCTGTGGCATCCATGAAACT; reverse, GAAGCACTTGCGGTGCACGAT.

Expression Vectors and Retrovirus Production. Full-length cDNA of mouse *Cbfβ* was purchased from TrueClone (catalog no. MC204426) and amplified by PCR. cDNA of mouse *Cbfβ* was inserted into an MSCV-IRES-GFP (MIG) plasmid. Retroviruses carrying empty vector (MIG-empty) or MIG-*Cbfβ* were produced using the Phoenix cell line. Virus supernatants were concentrated by centrifuging at 19,000 × *g*, 4 °C for 100 min. WT and $Cbf\beta^{-/-}$: *Tek-GFP/Cbfβ* fetal-liver MNCs were plated on retronectin-coated 24-well plates in induction medium with 10 ng/mL stem cell factor, 10 ng/mL IL-7, and 10 ng/mL Flit3-ligand. Concentrated virus supernatant was added into each well, and spin infection was performed by centrifuging at 1,000 × *g*, 32 °C for 100 min. The next morning, the medium was half changed, and second-spin infection was done. Three hours after second infection, cells were harvested and used for the transplantation assay. Infectious efficiency was confirmed by detecting GFP^{+} cells by flow cytometry.

Elispot. Elispot was done as previously described (39). Donor-derived B-1 or B-2 cells in the peritoneal cavity of recipient mice were sorted on a FACS Aria (Becton Dickinson), suspended in Elispot medium (RPMI 1640 medium with 10% FBS and 5×10^{-5} M β-mercaptoethanol), supplemented with 10 ng/mL IL-5, and cultured for 36–48 h in 96-well plates precoated with phosphorylcholine-conjugated keyhole limpet hemocyanin. Five hundred to 1,000 cells were distributed into an Elispot $MultiScreen_{HTS}$ Filter Plate (Millipore) precoated with anti-mouse IgM and cultured in Elispot medium overnight. After washing, alkaline phosphatase-conjugated goat anti-mouse IgM (Southern Biotech) was applied to each well and incubated for 45 min. After washing, spots were visualized using BCIP/NBTplus substrate as described (Mabtech).

Statistics. All experiments were completed in triplicate. The Student *t* test was used for statistical analysis.

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Mini Review

Protein Synthesis: More Than a House-Keeping Function in Hematopoietic Stem Cells

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Hematopoietic stem cell (HSC) self-renewal is tightly regulated by both extrinsic signaling pathways and intrinsic regulators [1]. Adding to this list, in a recent issue of *Nature*, Signer et al. (2014) show that protein synthesis also plays a critical role in regulating hematopoietic stem cell self-renewal [2].

HSCs reside in a hypoxic niche in the bone marrow and majority of HSCs are in the quiescent state [3-4]. In addition, HSCs have low mitochondrial activity [5-6] and may have relatively lower rate of ribosome biogenesis. Thus, quiescent HSCs can be identified by using the Pyronin Y/Hoechst staining. Hoechst is an exclusive DNA dye while Pyronin Y reacts with both DNA and RNA. In the presence of Hoechst, Pyronin Y reaction with DNA is blocked, and Pyronin Y stains RNA only. When cells are stained first with Hoechst 33342 and then with Pyronin Y it is possible to distinguish DNA from RNA. Furthermore, quiescent cells, which are arrested in G0 phase, have lower level of RNA compared to active cells (G1 phase). The co-staining with the RNA dye Pyronin Y allows for the separation of G0 and G1 cell populations [7]. However, the protein synthesis rate in HSCs has not been determined.

It has been challenging to measure protein synthesis rate *in vivo*. Recently, Liu and colleagues developed a novel fluorogenic assay by using an alkyne analog of puromycin, O-propargyl-puromycin (OP-Puro), which can incorporate into newly synthesized peptides [8]. By injecting OP-Puro into mice, one can quantitate the protein synthesis rate in cells by using the flow cytometry analysis. Signer and colleagues utilized this novel method and found that HSCs and multi-potent progenitor cells (MPPs) synthesize less protein per hour compared to other hematopoietic cells, including common myeloid progenitors (CMPs), granulocyte and macrophage progenitors (GMPs) and terminally differentiated cells [2]. Interestingly, the reduced protein synthesis is not due to the differences in cell cycle status, cell size, ribosomal RNA content between HSCs and more mature cells.

To examine whether the reduced protein synthesis rate is important for HSC function, the authors utilized the *RPL24*$^{Bst/+}$ mice, which harbors a loss-of-function mutation in the ribosomal protein gene *RPL24*. While these mice appear normal, there is a 30% decrease of protein synthesis rate [9]. RPL24 deficiency impairs both HSC self-renewal and proliferation potential in a cell-autonomous manner [2]. On the other hand, increased protein synthesis rate caused by *Pten* deletion depletes HSCs and promotes leukemogenesis [10]. Strikingly, crossing *RPL24*$^{Bst/+}$ mice with *Pten*$^{fl/fl}$*-Mx1-Cre* mice restores HSC function and brings the protein synthesis rate to normal levels [2], indicating that a delicate balance in protein synthesis is critical for HSC function and tumor suppression (Figure 1). Mutations in ribosomal genes and other genes that are important for protein synthesis have been implicated in human diseases. While increased protein synthesis promotes development of certain cancers, defective ribosomal function causes ribosomopathies [11]. Ribosomopathies are human disorders of ribosome dysfunction, in which genetic abnormalities cause impaired ribosome biogenesis and function, resulting in specific clinical phenotypes [11].

Impaired ribosomal function activates the p53 pathway, leading to cell cycle arrest and/or apoptosis [12]. However, the p53 pathway is not activated in RPL24Bst/+HSCs [2]. How protein synthesis is regulated in HSCs is largely unknown. It is possible that other mutations that affect ribosome biogenesis or translation will also reduce protein synthesis in HSCs. Indeed, reduced RPS19 or RPL11 expression in mouse erythroblast deregulates translation initiation of specific transcripts [13]. Investigation of whether other disease-associated ribosomal protein gene mutations affect protein synthesis will improve our understanding of how protein synthesis is regulated in HSCs.

It appears that different ribosomal proteins have distinct biological functions other than constituting ribosomes. In hematopoietic cells, RPS14 haploinsufficiency and RPS19 mutations are associated with 5-q syndrome and Diamond-Blackfan Anemia (DBA), respectively [14-15]. The common mechanism of the two diseases is activation of the p53 pathway [11]. However, unlike the RPL24Bst/+ mutation, these mutations specifically affect erythroid cells while sparing other lineages [14-15]. There are many ribosomal protein gene mutations have been identified in DBA patients, including RPS24, RPS17, RPL35A, RPL5 and RPL11 [11]. Although these mutations share some common manifestations including anemia, macrocytosis,

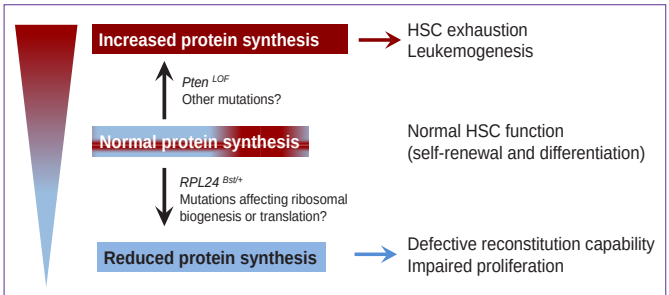

Figure 1: A balanced protein synthesis rate is important for HSC function. Hematopoietic stem cells (HSCs) require appropriate level of protein synthesis to balance self-renewal and differentiation. Reduced protein synthesis impairs the ability for HSCs to self-renew, whereas increased protein synthesis depletes HSCs and promotes leukemogensis.

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and absence of erythroid precursors, different ribosomal protein gene mutations display different phenotypes. For example, RPL5 gene mutations are associated with a higher frequency of physical abnormalities, including cleft lip and/or palate, whereas mutations in RPL11 had more isolated thumb abnormalities compared with patients with RPS19 mutations [16]. It will be interesting to investigate the distinct functions of different ribosomal proteins.

Overall, Signer and colleagues demonstrate that the protein synthesis in HSCs is tightly controlled. However, the exact mechanism underlying this process remains elusive. It is not clear why HSCs require lower rate of protein synthesis. Whether lower rate of protein synthesis is required for HSC self-renew awaits further investigation. Another interesting question is that whether there is an overall reduction of all protein species or only the synthesis of selective protein species is reduced in HSCs. Signer and colleagues report that HSCs and multi-potent progenitor cells (MPPs) have lower level of 4EBP1 phosphorylation compared to other hematopoietic cells, suggesting that the cap-dependent translation rate is low in HSCs and MPPs. How about cap-independent translation in HSCs and MPPs? Is cap-independent translation rate also low in HSCs? Reduced RPS19 and RPL11 expression in mouse erythroblasts impair the internal ribosomal entry site (IRES)–mediated translation of mRNAs [13]. It will be interesting to investigate whether HSCs also shut down IRES-dependent translation of mRNAs.

While it is clear that protein synthesis is important for HSC function, many questions remain unanswered. What are the links between hypoxic environment and mitochondrial activity? What is the relationship between hypoxic environments and stem cells with respect to energy production and therefore protein production? Why would decreased protein production be important for a stem cell (or just HSCs)? What is the link between potency and protein production? Further research should be directed to address these important questions and uncover the full regulatory network involved in HSC regulation. Hematopoietic stem cell is the best characterized somatic stem cell so far and has become a model for stem cell research [1]. It is highly possible that a balanced protein synthesis rate is essential for the maintenance of other stem cells as well.

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Phosphatase of regenerating liver in hematopoietic stem cells and hematological malignancies

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The phosphatases of regenerating liver (PRLs), consisting PRL1, PRL2 and PRL3, are dual-specificity protein phosphatases that have been implicated as biomarkers and therapeutic targets in several solid tumors. However, their roles in hematological malignancies are largely unknown. Recent findings demonstrate that PRL2 is important for hematopoietic stem cell self-renewal and proliferation. In addition, both PRL2 and PRL3 are highly expressed in some hematological malignancies, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma (MM) and acute lymphoblastic leukemia (ALL). Moreover, PRL deficiency impairs the proliferation and survival of leukemia cells through regulating oncogenic signaling pathways. While PRLs are potential novel therapeutic targets in hematological malignancies, their exact biological function and cellular substrates remain unclear. This review will discuss how PRLs regulate hematopoietic stem cell behavior, what signaling pathways are regulated by PRLs, and how to target PRLs in hematological malignancies. An improved understanding of how PRLs function and how they are regulated may facilitate the development of PRL inhibitors that are effective in cancer treatment.

Keywords: hematopoietic stem cell, hematological malignancy and therapeutic target, oncogenic signaling, PRL, PTP4A, self-renewal

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Protein tyrosine phosphatases (PTPs) control signaling pathways that are important for cell proliferation, adhesion and migration.[1,2] Some PTPs, including PTEN and SHP2, are dysregulated in hematological malignancies.[3,4] The tumor suppressor PTEN is mutated or deleted in leukemia and lymphoma, resulting in constitutive activation of the PI3K/AKT pathway that promotes leukemic cell proliferation and survival.[5-8] SHP2 (encoded by *PTPN11*) is an oncoprotein and *PTPN11* mutations have been implicated in the pathogenesis of several hematological malignancies, including acute myeloid leukemia (AML) and juvenile myelomonocytic leukemia (JMML).[9,10] Accumulating evidence indicate that oncogenic PTPs may serve as promising therapeutic targets in human cancer.[3,4]

The PRL (Phosphatase of Regenerating Liver) phosphatases constitute a novel class of prenylated phosphatases (PRL1, 2, and 3) that share a high degree (>76%) of sequence identity.[11-14] This family of phosphatases are also known as protein tyrosine phosphatase 4As (PTP4As). The PRLs are relatively small proteins of about 20 kDa. In addition to the phosphatase domain, there are no regulatory domains except that all PRLs contain a consensus C-terminal prenylation motif CaaX, which is important for their localization to the plasma membrane and early endosomal compartments.[11-14] *PRL1* was originally identified as an immediate early gene induced during liver regeneration after partial hepatectomy.[15] Subsequently, *PRL1* as well as the closely related *PRL2* and *PRL3* were found to be elevated in numerous tumor cell lines, and cells expressing high levels of PRLs exhibit enhanced proliferation and anchorage-independent growth.[16-22] Unlike most protein phosphatases that counteract the activity of protein kinases, the PRLs play a positive role in signaling and possess oncogenic properties.[11-14] Among the PRLs, PRL3 abnormal expression has been shown to be associated with poor

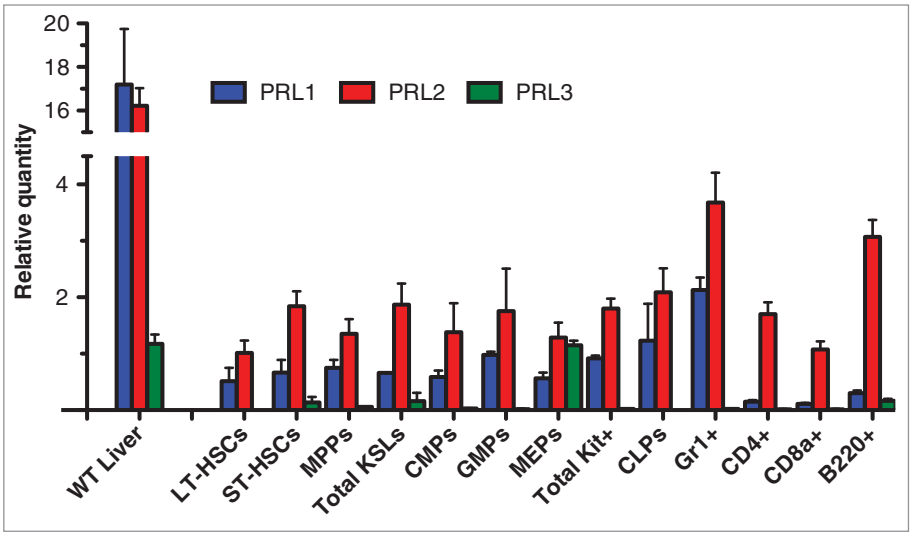

Figure 1. Expression of PRLs in hematopoietic cells. Real-time RT-PCR analysis of *PRL* mRNAs in long-tern HSCs (LT-HSCs), short-term HSCs (ST-HSCs) and representative committed progenitors and differentiated cells. MPPs, multi-potential progenitors; CLPs, common lymphoid progenitors; CMPs, common myeloid progenitors; MEPs, megakaryocyte-erythroid progenitors; GMPs, granulocyte-macrophage progenitors.

prognosis for patients with ovarian and breast cancers.[23,24] In addition, elevated PRL3 expression has been implicated as a biomarker for colorectal cancer.[25,26] Compared to PRL3, the roles of PRL1 and PRL2 in human cancer are largely unknown. There are several excellent reviews about PRLs and human cancer.[11-14] Here, we will summarize recent findings related to the emerging roles of PRLs in normal and malignant hematopoiesis and discuss their potential in cancer therapy.

PRLs in Hematopoietic Stem Cells

In order to maintain hematopoietic homeostasis throughout the life of an animal, the hematopoietic stem cell (HSC) pool must be maintained through the process of self-renewal.[27] HSC self-renewal requires the integration of survival and proliferation signals to maintain an undifferentiated state. This demands a complex crosstalk between extrinsic signals from the microenvironment and the cell-intrinsic regulators of HSCs.[28,29] Given that PRLs are known to modulate several signaling pathways in normal and cancer cells,[11-14] it is possible that PRLs are also important for hematopoietic stem cell maintenance.

PRL2 regulates haematopoietic stem cell self-renewal

While *PRL1* and *PRL2* are broadly expressed in adult tissues including liver,[30,31] the expression pattern of these *PRLs* in the hematopoietic system is unknown. We found that *PRL1* and *PRL2* are ubiquitously expressed in the hematopoietic compartment, whereas *PRL3* expression is most pronounced in megakaryocyte-erythroid progenitor cells (MEPs) [**Figure 1**]. *PRL2* is highly expressed in hematopoietic cells and its expression is enriched in differentiated cells compared to long-term HSCs and committed progenitor cells (**Fig. 1**), suggesting that it could play an important role in lineage commitment.[32] To define the role of PRL2 in regulating hematopoietic stem cell self-renewal, we performed serial bone marrow transplantation assays and found that loss of *PRL2* impairs the ability of haematopoietic stem cells to repopulate the lethally irradiated recipient mice. The decreased self-renewal capability is not due to homing defects or increased HSC apoptosis, demonstrating that PRL2 regulates hematopoietic stem cell self-renewal in a cell autonomous manner.[32]

PRL2 regulates hematopoietic stem cell proliferation

Ectopic expression of *PRL2* in nontumorigenic cells has been shown to enhance cell cycle progression and cell proliferation,[20,33] indicating PRL2 may also promote hematopoietic stem and progenitor cell (HSPC) proliferation. This is indeed the case. PRL2 null HSPCs are more quiescent and less proliferative.[32] Both p21 and p57 have been shown to regulate HSPC proliferation[34,35] and we observed decreased expression of p21 and p57 in PRL2 null hematopoietic stem and progenitor cells;[32] therefore, PRL2 plays an important role in promoting HSPC proliferation, at least in part, by controlling the level of cell cycle regulators. How PRL2 regulates the expression of these cell cycle regulators is not clear.

PRL2 regulates lymphoid differentiation

The *PRL2*$^{-/-}$ mice have smaller spleen and thymus compared with wild type mice.[32] Loss of PRL2 also decreases lymphocyte count in peripheral blood.[32] In serial bone marrow transplantation assays, we found that the percentage of donor-derived T cells was significantly lower in mice transplanted with *PRL2* null cells than that with wild-type cells.[32] Analysis of T cell development reveals that loss of PRL2 blocks T cell differentiation at the DN2 stage, indicating that PRL2 is important for expansion of T cell progenitors (M.K., Y.L., unpublished data).

Signaling Pathways Regulated by PRLs

Although members of the PRL phosphatase family have crucial roles in various cellular processes, including cell proliferation, migration and invasion, the physiological functions of individual PRLs are poorly understood owing to the lack of understanding of the genuine substrates of the perspective PRLs.[11-14] Accumulating evidence indicates that PRLs potentially regulate several signaling pathways essential for HSPC proliferation and survival.

SCF/KIT signaling

Stem cell factor (SCF) is a dimeric molecule that exerts its biological

functions by binding to and activating the receptor tyrosine kinase KIT.[36] KIT is highly expressed in HSCs and SCF/KIT signaling plays a critical role in HSC maintenance.[37-46] Studies in mice with either partial or complete loss-of-function KIT mutations, have revealed severe hematopoietic deficiencies of multiple lineages, as well as an important role of KIT in sustaining rapidly cycling HSCs post transplantation when competing with wild-type HSCs.[41-46] Despite the wealth of knowledge on SCF/KIT signaling, it is poorly understood how KIT signaling is regulated in HSCs.[36] As *PRL2* null hematopoietic progenitor cells show decreased colony formation in serial replating assays,[32] it is possible that PRL2 regulates cytokine signaling in hematopoietic progenitor cells. While *PRL2* null hematopoietic progenitor cells proliferate normally in response to IL-3 or G-CSF stimulation, these cells are less proliferative in response to SCF stimulation. Furthermore, *PRL2* null hematopoietic progenitor cells show decreased colony formation in response to increased concentrations of SCF compared with wild-type cells.[32] Thus the hematopoietic defects seen in the *PRL2* null mice recapitulate several hematopoietic phenotypes of KIT mutant mice. [42-46] Interestingly, PRL2 is also found important for SCF/KIT signaling during spermatogenesis.[47] Importantly, the ability for PRL2 to enhance SCF/KIT signaling depends on its phosphatase activity,[32] indicating that KIT may be a direct target of PRL2 in hematopoietic stem and progenitor cells. Thus, PRL2 functions as a novel regulator of SCF/KIT signaling.

FLT3 signaling

FLT3 is a receptor tyrosine kinase with important roles in hematopoietic stem/progenitor cell survival and proliferation.[48,49] It is mutated in about 1/3 of acute myeloid leukemia (AML) patients, either by internal tandem duplications (ITD) of the juxtamembrane domain or by point mutations usually involving the kinase domain (KD). Both types of mutation constitutively activate FLT3.[50,51] PRL3 has been shown to participate in FLT3-ITD signaling in AML cells.[52,53] PRL3 is downstream of FLT3 and its overexpression enhances the activation of both MAPK/ERK and STAT pathways.[52,53] We also found that PRL2 is important for FLT3-ITD-mediated ligand-independent growth of hematopoietic stem and progenitor cells (M. K. and Y. L., unpublished data). While both PRL2 and PRL3 are involved in oncogenic FLT3 signaling in hematopoietic cells, how PRLs mediate FLT3-ITD signaling is largely unknown.

PI3K/PTEN/AKT signaling

Among the signal transduction pathways that have attracted considerable attention as possibly being involved in HSC maintenance is the phosphoinositide 3-kinase (PI3K)-AKT pathway.[5-7] As an antagonist of the PI3K pathway, PTEN has been implicated as a regulator of HSC self-renewal and loss of PTEN in the HSC compartment results in hyperproliferation of hematopoietic stem and progenitor cells.[8] In mouse placenta, PRL2 promotes cell proliferation by activating the AKT kinase through downregulation of the tumor suppressor PTEN.[54] It appears that PRL2 overexpression enhances the degradation of PTEN protein in these cells, indicating that PRL2 regulates the stability of PTEN in placenta.[54] In addition, we observed that *PRL2* null hematopoietic progenitor cells show decreased phosphorylation of AKT and ERK1/2 in steady state. To investigate how PRL2 regulates PI3K/AKT signaling in hematopoietic progenitor cells, we examined the level of PTEN in *PRL2* null HSPCs and observed elevation of PTEN protein, whereas PTEN RNA level was identical between *PRL2* wild-type and null cells. Therefore, it is possible that PRL2 regulates the stability and turnover of PTEN protein in hematopoietic stem and progenitor cells as well. PTEN has been shown to regulate cell proliferation and cancer progression in a dosage-dependent manner and a slight reduction in PTEN levels dictates cancer susceptibility.[55-57] Thus, it is likely that increased level of PTEN we observed contributes to the decreased activation of AKT and ERK1/2 signaling pathways in *PRL2* null HSPCs. Given that PRL3 can downregulate PTEN in HeLa cells to promote epithelial-mesenchymal transition,[58] PRL3 may also regulate PTEN levels in hematopoietic cells.

Ras/MEK/ERK signaling

Growth factors and mitogens use the Ras/Raf/MEK/ERK signaling cascade to transmit signals from their receptors to regulate gene expression and prevent apoptosis.[59] Both K-RAS and N-RAS are mutated in hematological malignancies and activation of the Raf/MEK/ERK pathway is usually associated with proliferation and drug resistance of hematopoietic cells.[60] Expressing oncogenic Ras (KrasG12D or NrasG12D) in HSCs activates ERK1/2 signaling and enhances HSC proliferation and self-renewal.[61-64] Mutations that increase HSC proliferation tend to reduce competitiveness and self-renewal potential, raising the question of how HSCs expressing oncogenic Ras can outcompete wild-type HSCs. Li and colleagues found that oncogenic Ras (NrasG12D) had a bimodal effect on HSCs, increasing the frequency with which some HSCs divide and reducing the frequency with which others divide. Thus, one signal can therefore increase HSC proliferation, competitiveness and self-renewal through bimodal effects on HSC gene expression, cycling and reconstituting potential.[65] Given that ectopic PRL expression activates the ERK pathway, [11-14] it is likely that increased association of PRLs with RAS may activate the MEK/ERK signaling pathway in hematopoietic stem and progenitor cells (HSPCs) and enhance their proliferation and survival [**Figure 2**].

JAK/STAT signaling

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway is central to signaling by cytokine receptors. These receptors, including receptors for erythropoietin, thrombopoietin, most interleukins and interferon, transmit anti-apoptotic, proliferative and differentiation signals in hematopoietic cells.[66-67] The JAK-STAT pathway is important for hematopoiesis and several activating mutations in JAK proteins have recently been described as underlying cause of blood disorders.[66-67] One of the best studied examples is the

JAK2 V617F mutant which is found in 95% of polycythemia vera patients and 50% of patients suffering from essential thrombocythemia and primary myelofibrosis.[68] JAK2 V617F promotes myeloid progenitor populations expansion and skews their differentiation toward the erythroid lineage.[69] In addition, STAT5 activation is important for HSC expansion as well as amplification of the erythroid compartment.[67] Ectopic PRL3 expression enhances the phosphorylation of STAT5. [52] Furthermore, Park and colleagues demonstrated that STAT5 is a transcriptional activator of PRL-3 expression,[53] suggesting a feed-forward loop in STAT5 activation in hematopoietic cells expressing PRL3. We also found that PRL2 overexpression in Baf3 cells enhances STAT5 activation (M. K., Y.L., unpublished data), providing further evidence that PRLs regulate the JAK/STAT pathway.

In addition to signaling pathways, PRLs may also utilize other mechanisms to promote leukemia cell proliferation and survival. Zeng and colleagues recently demonstrated that ectopic PRL3 expression upregulates Leo1 expression in AML cells.[70] Leo1 is a component of RNA polymerase II-associated factor (PAF) complex[71] and loss of Leo1 leads to downregulation of SOX2 and SOX4, which are potent oncogenes in myeloid transformation.[70] Thus, Leo1 is an important mediator of PRL3 oncogenic activities in AML.

PRLs in Hematological Malignancies

PRLs are highly expressed in human cancer cells, including ovarian, lung, breast and liver cancers.[11-14] Recent findings suggest that PRLs may play important roles in the pathogenesis of hematological malignancies.

PRLs in myeloid malignancies

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. It usually occurs around age 60 with no identifiable cause and carries a very poor prognosis, with most patients living less than 18 months.[72,73] Unfortunately, little progress has been made in treating AML over the past 4 decades.[73] Clearly, new treatment strategies are urgently needed.

AML is a highly heterogeneous disease with multiple signaling pathways contributing to its pathogenesis.[74,75] Gain-of-function mutations in KIT receptor in humans are associated with gastrointestinal stromal tumors (GIST), systemic mastocytosis (SM), and AML.[76-78] These mutations results in altered substrate recognition and constitutive tyrosine autophosphorylation leading to promiscuous and constitutive signaling.[77-79] Consequently, cell lines and primary bone marrow (BM) cells that express the oncogenic KIT mutant (KIT/D814V) demonstrate ligand-independent proliferation *in vitro* and myeloproliferative disease (MPD) *in vivo*.[78-83] However, the intracellular signals that contribute to mutant KIT-induced MPD are not known. We discovered that loss of *PRL2* decreased the ability of KIT/D814V mutant in promoting hematopoietic progenitor cell proliferation, demonstrating that PTRL2 is an important effector molecule of oncogenic KIT signaling in hematopoietic stem and progenitor cells and positively contributes to KIT/D814V-induced hematopoietic progenitor hyperproliferation.[32] While *PRL2* mRNA is highly expressed in pediatric acute myeloid leukemia cells,[84] its role in malignant hematopoiesis is largely unknown. We found that PRL2 is overexpressed in some human AML cell lines and genetic inhibition of PRL2 decreases the proliferation of human AML cells (M.K. and Y. L., unpublished data), indicating that PRL2 may play a pathological role in AML.

FMS-like tyrosine kinase 3 (FLT3) is the most frequently mutated gene in AML.[48,49] Many studies have shown that AML patients with FLT3-ITD

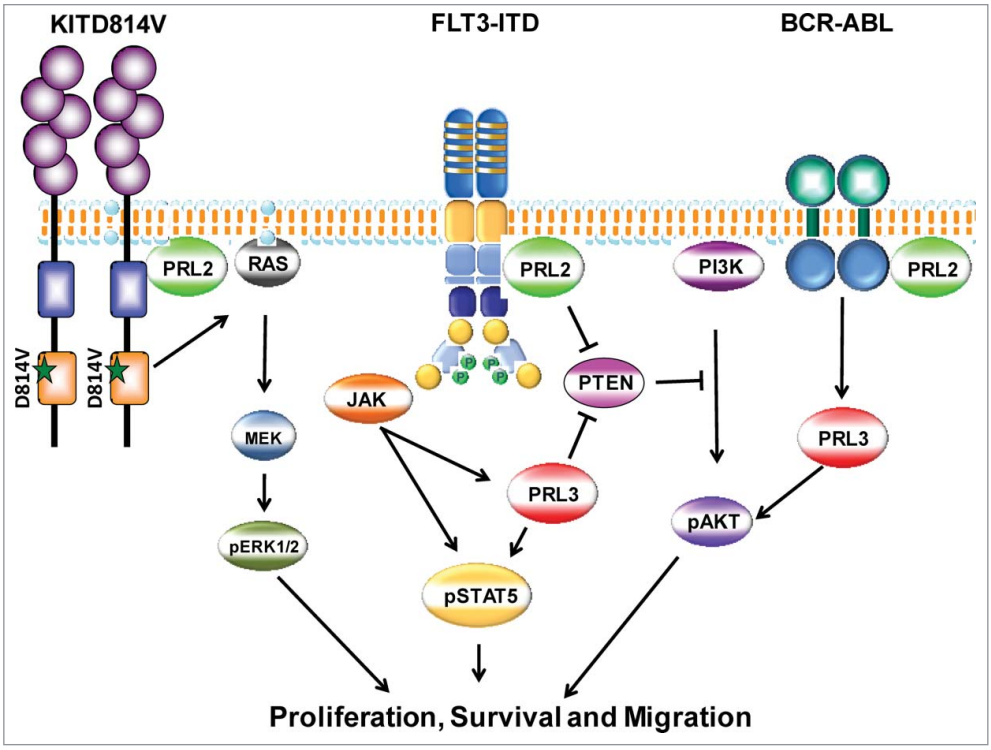

Figure 2. PRLs regulates oncogenic signaling pathways in hematopoietic stem and progenitor cells. A schematic model shows that both PRL2 and PRL3 mediate oncogenic signaling, including KITD814V, FLT3-ITD, BCR-ABL, PI3K/AKT, Ras/MEK/ERK and JAK/STAT. Accumulating evidence indicate that elevated PRL expression may contribute to the pathogenesis of hematological malignancies and targeting PRLs with small molecule inhibitors may improve blood cancer treatment.

mutations have poor cure rates due to relapse.[48-51] This has led to the development of a number of small molecule tyrosine kinase inhibitors (TKI) with activity against FLT3; however, the clinical activity of early inhibitors was limited by a lack of selectivity, potency and unfavorable pharmacokinetic properties.[48-51] PRL3 is a downstream target of FLT3-ITD in human AML cells and knockdown of PRL3 decreases the proliferation of AML cells.[52,53] High PRL3 mRNA expression is associated with FLT3-ITD mutations and poor prognosis in AML patients with normal karyotype.[52,53,85] Internal tandem duplication of FMS-like tyrosine kinase (FLT3-ITD) is well known to be involved in acute myeloid leukemia (AML) progression, but FLT3-ITD-negative AML cases account for 70% to 80% of AML, and the mechanisms underlying their pathology remain unclear.[48-51] PRL3 is upregulated in 40% of FLT3-ITD-negative AML patients. In addition, PRL3 expression level correlates with poor survival of the AML patients, and the AML relapses accompany with re-upregulation of PRL3,[86] suggesting that PRL3 is important for the proliferation and survival of both FLT3-ITD-positive and FLT3-ITD-negative AML cells.[52,53,85,86]

Chronic myeloid leukemia (CML) is caused by the t(9;22) chromosomal translocation that results in the expression of the fusion tyrosine kinase BCR-ABL. Tyrosine kinase inhibitor (TKI) therapy has led to long-term remissions, but patients remain BCR-ABL+.[87,88] While BCR-ABL kinase is active in CML stem cells, TKIs inhibit the kinase activity without affecting CML stem cell survival.[87,88] A better understanding of the BCR-ABL signaling network may lead to novel therapy that can eliminate CML stem cells. PRL3 is upregulated in human CML cell lines and primary CML patient samples. In addition, PRL3 appears to be a downstream target of BCR-ABL signaling as inhibiting BCR-ABL signaling decreases PRL3 expression.[89] How BCR-ABL signaling regulates PRL3 expression is not known. We found that PRL2 is highly expressed in human CML cell line K562 (M.K. and Y.L., unpublished data), indicating that PRL2 may involve in BCR-ABL signaling as well.

Multiple myeloma (MM) remains to be a hard-to-treat hematologic cancer in the era of new and effective therapeutic protocols. The hallmark of MM is a sequel to drug resistant phenotypes persisting initially or emerging in the course of treatment. Furthermore, the heterogeneous nature of multiple myeloma makes treating patients with the same drug challenging.[90,91] PRL3 is highly expressed in human myeloma cells and knockdown of PRL3 inhibits the migration but not the proliferation of human MM cells.[92] PRL3 is a metastasis-associated phosphatase in some human cancers and can reduce the number of focal adhesions and /or increase focal adhesion turnover to mediate cell invasion and motility.[24-26] While PRLs have positive role in the proliferation of myeloid leukemia cells, PRL3 appears to promote the migration of human MM cells. It is possible that the distinct microenvironment where cells reside may dictate the function of PRLs to promote proliferation or migration. In addition, PRLs may utilize different substrates to promote the pathogenesis of AML vs. MM.

PRLs in lymphoid malignancies

Acute lymphoblastic leukemia (ALL) is a heterogeneous group of hematologic malignancies that arise from clonal proliferation of immature lymphoid cells in the bone marrow, peripheral blood and other organs. There are approximately 3000 new adult cases diagnosed every year in the United States with a 5-year overall survival ranging from 22% to 50%. [93] T-cell acute lymphoblastic leukemia (T-ALL) is a malignant subtype of lymphoid leukemia accounting for 15% of pediatric and 25% of adult ALL cases.[94-96] The aggressive and unpredictable behavior of relapsed T-ALL presents a major clinical challenge, with >70% of children and >90% of adults unable to survive their relapsed disease.[95,96] The cytotoxic therapies used to treat primary T-ALL are often ineffective against relapsed T-ALL,[96,97] suggesting that a subset of cells have acquired mutations allowing them to survive treatment and drive relapse growth. Therefore, a detailed knowledge of the molecular and genetic changes that contribute to T-ALL relapse is urgently needed to design novel target therapies and improve leukemia treatment. Both PRL2 and PRL3 are upregulated in human T-ALL cells based on data from the Oncomine database and we found that PRL2 is important for oncogenic Notch-induced T-ALL in vivo (M.K.,Y.L., unpublished data). Thus, PRLs may be a potential therapeutic target in T-ALL.

The Philadelphia chromosome (Ph+) reflects a balanced reciprocal translocation between the long arms of chromosomes 9 and 22 [t(9;22)(q34;q11.2] involving the BCR and ABL genes. [98,99] The clinical management of patients with Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL) has been challenging primarily due to the aggressive nature of the disease and limited effective treatment options.[98,99] PRL3 is highly expressed in BCR-ABL positive ALL, which may be responsible for the aggressive phenotype of the disease.[100] *PRL2* is located on human chromosome 1p35,[11] a region often rearranged or amplified in malignant lymphoma and B-cell chronic lymphocytic leukemia (B-CLL).[101,102] Thus, it is possible that PRLs may play a pathological role in human lymphoid malignancies.

Targeting PRLs in Hematological Malignancies

PRLs have been validated as biomarkers and therapeutic targets in some human cancers.[11-14] Given that PRLs are upregulated in some hematological malignancies and important for the proliferation, survival and migration of malignant cells through enhancing oncogenic signaling pathways,[52,53,84-86,92,100] PRLs may be potential therapeutic targets in hematological malignancies.

Targeting PRLs with small molecule inhibitors

PRLs are attractive targets for developing inhibitors as anticancer therapeutics given their potentially pathological role in human cancer.[11-14,103] PRLs are characterized by low activity in vitro.[104] In addition, the catalytic clefts of PRLs seem to be shallow, suggesting a broad range of

specificity.[105,106] Thus, it has been challenging to identify small molecule inhibitors with a high degree of specificity to PRLs.[13,14] Several compounds, including pentamidine, rhodanine derivatives, and Ezrin, have been shown to inhibit PRL activities in some human cancer cell lines.[107-113] However, none of these PRL inhibitors have been used to inhibit PRLs activity in human hematological malignancies. It will be interesting to observe similar effects in hematological malignancies as seen in solid tumors.

The PRLs are unique among protein-tyrosine phosphatases in that they have a high propensity for trimer formation.[11-14] We found that PRL1 exists as a trimer in the crystalline state and the trimerization creates a large, bipartite membrane-binding surface in which the exposed C-terminal basic residues could cooperate with the adjacent prenylation group to anchor PRL1 on the acidic inner membrane.[114] We further demonstrated that PRL1 trimer formation is essential for PRL1-mediated cell growth and migration.[115] Therefore, screening small molecules that can disrupt PRL trimmer formation may represent a novel concept in targeting PRLs in human cancer.[14] Because of the high level of primary sequence identity between the PRL family members, identifying inhibitors specific to a certain PRL might be difficult.[11,12] Given that both PRL2 and PRL3 seem to involve in the growth of human AML cells,[52,53,84-86,92,100] an agent affecting all PRLs might be desirable in hematological malignancies.

Targeting PRLs with chimeric antibodies

Immunotherapy has been actively investigated as a novel therapeutic approach to target surface proteins in human cancer cells.[116,117] As PRLs are intracellular proteins,[11-14] it is difficult to utilize antibodies to target them. However, the Zeng group demonstrated that a PRL3 chimeric antibody can efficiently and specifically reduce the formation of PRL3 expressing metastatic tumors and has antitumor activity in a xenograft model of human cancer.[118-120] Furthermore, PRL3 chimeric antibody inhibits the growth of FLT3-ITD positive AML cells both *in vitro* and *in vivo*.[53] Thus, PRL-specific antibodies may be developed to treat human cancer, including hematological malignancies.[53,118-120]

Conclusions and Future Directions

In summary, PRL2 is important for hematopoietic stem cell self-renewal and proliferation. In addition, both PRL2 and PRL3 are highly expressed in some hematological malignancies, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML), multiple myeloma (MM) and acute lymphoblastic leukemia (ALL). Moreover, PRL deficiency impairs the proliferation and survival of leukemia cells through regulating oncogenic signaling pathways (**Fig. 2**). These studies indicate that PRLs may serve as potential biomarkers and therapeutic targets in hematological malignancies. However, many questions remain to be addressed for a more complete understanding of the importance of PRLs in the pathogenesis of hematological malignancies.

First, we need to evaluate whether PRL family members have overlapping or distinct functions in hematopoiesis. Given that PRL1, PRL2 and PRL3 share high sequence identity,[5-8] it is possible that PRL family members have overlapping functions in regulating hematopoietic stem cell behavior. We have generated knockout mice for PRL1, PRL2 and PRL3 (Z-Y.Z., unpublished data). Analyzing HSC behavior in mice deficient for 2 or 3 PRL family members will provide novel insight into the role of this still poorly understood family of protein tyrosine phosphatases in regulating hematopoietic stem cell self-renewal and terminal differentiation.

Second, it is important to elucidate the mechanism by which overexpression of PRLs contributes to the pathogenesis of hematological malignancies. PRLs are dual specificity protein phosphatases.[11-14] While PRLs have been show to interact with oncoproteins and tumor suppressors, including PTEN, p53 and Src, the substrates of PRLs are largely unknown. Many potential substrates and pathways have been proposed but none have been confirmed by dephosphorylation assays.[11-14] Given that PRLs are important for both myeloid and lymphoid malignancies, it is possible that PRLs target different substrates and/or pathways to promote leukemogenesis. Identifying substrates of PRLs is critical for understanding their roles in cancer progression and exploiting their potential as exciting new therapeutic targets for hematological malignancies. Phosphatase Substrate-trap methods and phospho-proteomic approaches can be used to identify potential PRL substrates in hematological malignancies.[121,122] By using these approaches, we have identified several potential PRL2 substrates in human AML cells and we are in the process of validating their role in AML (Z.Y.Z., unpublished data).

Third, it will be interesting to investigate whether high expression of PRLs can be used as biomarkers in hematological malignancies. PRLs are found significantly overexpressed in many human cancers, including colorectal cancer, breast cancer, lung cancer and gastric carcinoma, and have been implicated as useful biomarkers for disease progression and metastasis. [11-14] consistent with findings in solid tumors, high PRL3 expression correlates with poor prognosis and decreased survival of AML patients, [52,53,85,86] indicating that elevated PRLs expression may serve as novel biomarkers in hematological malignancies. Future works are needed to demonstrate that PRLs are important for the progression and the maintenance of other hematological malignancies.

Lastly, it should be tested whether PRL inhibitor treatment sensitizes drug-resistant leukemia cells to chemotherapies and/or tyrosine kinase inhibitors (TKIs) treatment. It has been shown that high PRL3 expression mediates drug-resistance in FLT3-ITD$^+$ AML cells.[52] Thus, it is possible that PRL inhibitor will synergize with chemotherapies or target therapies in the treatment of hematological malignancies, especially acute myeloid leukemia.

Fully addressing these questions will help to understand the function of PRLs in hematological malignancies and provide solid evidence for designing novel strategies for cancer treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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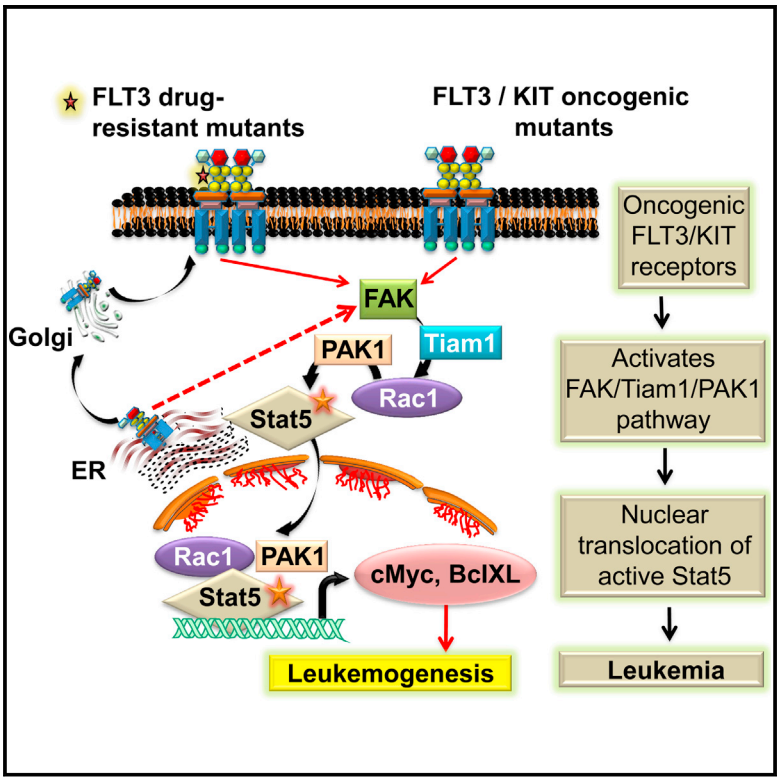
Cell Reports

Regulation of Stat5 by FAK and PAK1 in Oncogenic FLT3- and KIT-Driven Leukemogenesis

Graphical Abstract



Highlights

FAK/Tiam1/Rac1/PAK1 regulate active Stat5 downstream from oncogenic KIT and FLT3

FAK/Tiam1/PAK1 inhibition prolongs survival of mice harboring KIT and FLT3 mutations

AC220-resistant mutants of FLT3 are sensitive to inhibition by FAK/Tiam1/PAK1 axis

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In Brief

A significant impediment in treatment of leukemia, induced by oncogenic FLT3 and KIT receptors, is inadequate understanding of critical signaling pathways that lead to the development of this disease. In this study, Chatterjee et al. show an essential role of FAK/Tiam1/Rac1/PAK1 pathway in regulating nuclear translocation of Stat5 leading to leukemogenesis, in the context of oncogenic mutations of FLT3 and KIT, and provide multiple potential therapeutic targets to treat leukemia.

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Cell Reports
Article

Regulation of Stat5 by FAK and PAK1 in Oncogenic FLT3- and KIT-Driven Leukemogenesis

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SUMMARY

Oncogenic mutations of FLT3 and KIT receptors are associated with poor survival in patients with acute myeloid leukemia (AML) and myeloproliferative neoplasms (MPNs), and currently available drugs are largely ineffective. Although Stat5 has been implicated in regulating several myeloid and lymphoid malignancies, how precisely Stat5 regulates leukemogenesis, including its nuclear translocation to induce gene transcription, is poorly understood. In leukemic cells, we show constitutive activation of focal adhesion kinase (FAK) whose inhibition represses leukemogenesis. Downstream of FAK, activation of Rac1 is regulated by RacGEF Tiam1, whose inhibition prolongs the survival of leukemic mice. Inhibition of the Rac1 effector PAK1 prolongs the survival of leukemic mice in part by inhibiting the nuclear translocation of Stat5. These results reveal a leukemic pathway involving FAK/Tiam1/Rac1/PAK1 and demonstrate an essential role for these signaling molecules in regulating the nuclear translocation of Stat5 in leukemogenesis.

INTRODUCTION

Acute myeloid leukemia (AML) is a lethal disease characterized by uncontrolled growth of myeloid cells and is predominantly a disease of the elderly. Little progress has been made in terms of standard-of-care treatment for AML, which has essentially remained the same over decades. Long-term survival is observed in ~30% of younger patients and ~5% of older patients greater than 60 years of age. Internal tandem duplications (ITD), in-frame insertions, or duplication of amino acids near the juxtamembrane domain of FLT3 have been observed in ~25%–30% of all AML patients and confer a poor prognosis (Kottaridis et al., 2001). Likewise, Gleevec-resistant activation loop mutations of KIT (KITD816V) are found in a number of patients with core-binding factor-AML and ~95% patients with systemic mastocytosis (SM) and confer poor overall survival (Beghini et al., 2004). Both FLT3ITD and KITD816V receptors are constitutively phosphorylated (Kiyoi et al., 2002; Spiekermann et al., 2003) and induce growth in a ligand-independent manner. Whereas effort has been devoted to the development of FLT3 and KIT inhibitors, as single agents, the efficacy of these inhibitors is limited and in some cases results in drug resistance (Smith et al., 2012). Given that direct targeting of FLT3ITD or KITD816V has met with little success, signaling pathways downstream from FLT3ITD/KITD816V provide attractive alternate targets for treating hematologic malignancies involving these receptors.

Overexpression of focal adhesion kinase (FAK) in up to 50% of AML-patient-derived cells, but not in normal cells, has been observed, and FAK is hyperphosphorylated on Y397 in a number of patients. FAK+ AML cells show greater migration and resistance to daunorubicin compared to FAK− cells, and FAK expression correlates with high blast cell counts, early death, and shorter survival rate (Despeaux et al., 2011; Recher et al., 2004; Li and Hua, 2008). Presence of phosphorylated (p) pStat5 in newly diagnosed AML patients is also associated with poor overall survival (Brady et al., 2012). Constitutive activation of pStat5 is observed in 100% of SM patients bearing the KITD816V mutation (Baumgartner et al., 2009). A strong correlation between the presence of pStat5 and FLT3ITD mutations is seen in AML patients, and FLT3ITD expression results in constitutive Stat5 phosphorylation (Obermann et al., 2010; Spiekermann et al., 2003; Choudhary et al., 2005, 2007). Mutating the binding sites for Stat5 in the FLT3ITD abrogates the development of myeloproliferative neoplasms (MPNs) (Rocnik et al., 2006). Taken together, studies suggest that FLT3ITD/KITD814V, FAK, and Stat5 may be involved in regulating a critical pathway in AML and MPNs; however, the relationship between these signaling molecules in the context of leukemogenesis is not fully understood. Importantly,

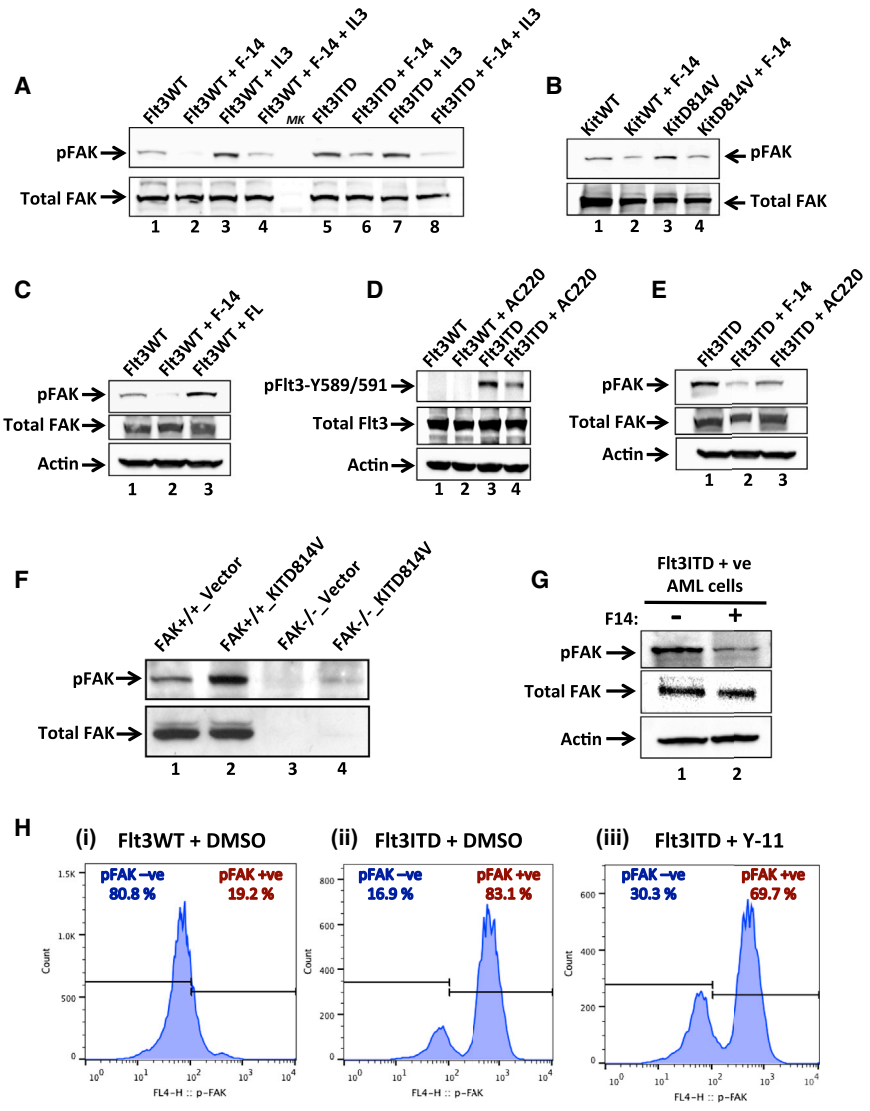

Figure 1. FAK Is Constitutively Phosphorylated in FLT3 and Activating KITD814V Oncogene-Bearing Cells

(A) Serum-starved 32D cells expressing FLT3ITD and FLT3WT were treated with DMSO (lanes 1 and 5), F-14 (lanes 2 and 6), IL-3 (lanes 3 and 7), or F-14 followed by IL-3 (lanes 4 and 8). An equal amount of protein was subjected to western blot analysis and probed with phospho-FAK (Y397) antibody (n = 3). "MK" denotes lane with protein ladder.

(B) 32D cells expressing KITWT or KITD814V were treated with F-14 (n = 2) and analyzed as described in (A).

(C) 32D cells expressing FLT3WT were treated with F-14 (lane 2) or stimulated with FLT3 ligand (FL) (lane 3) and analyzed as described in (A).

(D) 32D cells expressing FLT3WT or FLT3ITD were treated with FLT3ITD inhibitor AC220 and analyzed for activated FLT3 (pY589/591).

(E) 32D cells expressing FLT3ITD were treated with F-14 (lane 2) or with AC220 (lane 3; n = 2) and analyzed as above.

(F) Lysates from primary *FAK*−/−-deficient or WT BM cells expressing KITD814V or empty vector were analyzed for activated FAK.

(G) FLT3ITD+ve AML patient sample was analyzed for activated FAK (n = 2).

(H) 32D cells expressing FLT3ITD were treated with Y-11 and subjected to flow cytometry analysis. The percentage of cells show activated FAK under basal conditions in FLT3WT (i), FLT3ITD vehicle-treated cells (ii), and FLT3ITD treated with Y-11 (iii). n = 2.

although Stat5 has been implicated in regulating several hematologic malignancies, how precisely activation of Stat5 is regulated in the cytosol or in the nucleus of leukemic cells and what are the signaling molecules involved in its nuclear import in the context of AML or MPN remains unclear. Here, we reveal a leukemic pathway involving FAK/Tiam1/Rac1/PAK1 and demonstrate an essential role for these signaling molecules in regulating the nuclear translocation of Stat5 in leukemogenesis.

RESULTS

FAK Is Constitutively Phosphorylated in FLT3ITD- and KITD814V-Expressing Cells

32D cells expressing the wild-type (WT) FLT3 or KIT receptor (FLT3WT or KITWT) or its oncogenic version (FLT3ITD or KITD814V) were starved and treated with a FAK-specific inhibitor F-14 (Golubovskaya et al., 2008). Enhanced activation of FAK was observed in FLT3ITD-bearing cells compared to controls (Figure 1A, lane 1 versus 5), which was inhibited in the presence of F-14 (Figure 1A, lane 6 versus 5). Similar results were observed in cells expressing KITWT and KITD814V receptors (Figure 1B). To assess if activation of FAK was restricted to oncogenic FLT3- and KIT-receptor-expressing cells, same cells were stimulated with interleukin (IL)-3 to activate the IL-3 receptor and analyzed for FAK activation. As seen in Figure 1A (lane 3), IL-3 stimulation also resulted in activation of FAK, which was inhibited in the presence of F-14 (Figure 1A, lane 4). Similar results were observed upon treatment of cells with FLT3 ligand (FL) (Figure 1C, lane 1 versus 3). To assess the direct involvement of FLT3ITD in FAK activation, cells were treated with AC220, a potent FLT3ITD inhibitor (Smith et al., 2012). Treatment of FLT3ITD cells with AC220 inhibited the activation of FLT3ITD (Figure 1D, lane 3 versus 4) and also resulted in reduced FAK activation (Figure 1E, lane 1 versus 3). To rule out the nonspecific effects of F-14 on FAK inhibition, we utilized a genetic approach. WT bone marrow (BM) cells expressing KITD814V demonstrated increased levels of active FAK compared to controls, whereas *FAK*−/− BM cells showed absence of FAK expression (Figure 1F). FLT3ITD+ve AML-patient-derived cells also demonstrated constitutive FAK activation, which was inhibited in the presence of F-14 (Figure 1G). Figures 1D and S8D show the expression of total FLT3 and KIT receptors. We also performed intracellular staining to determine the effect of

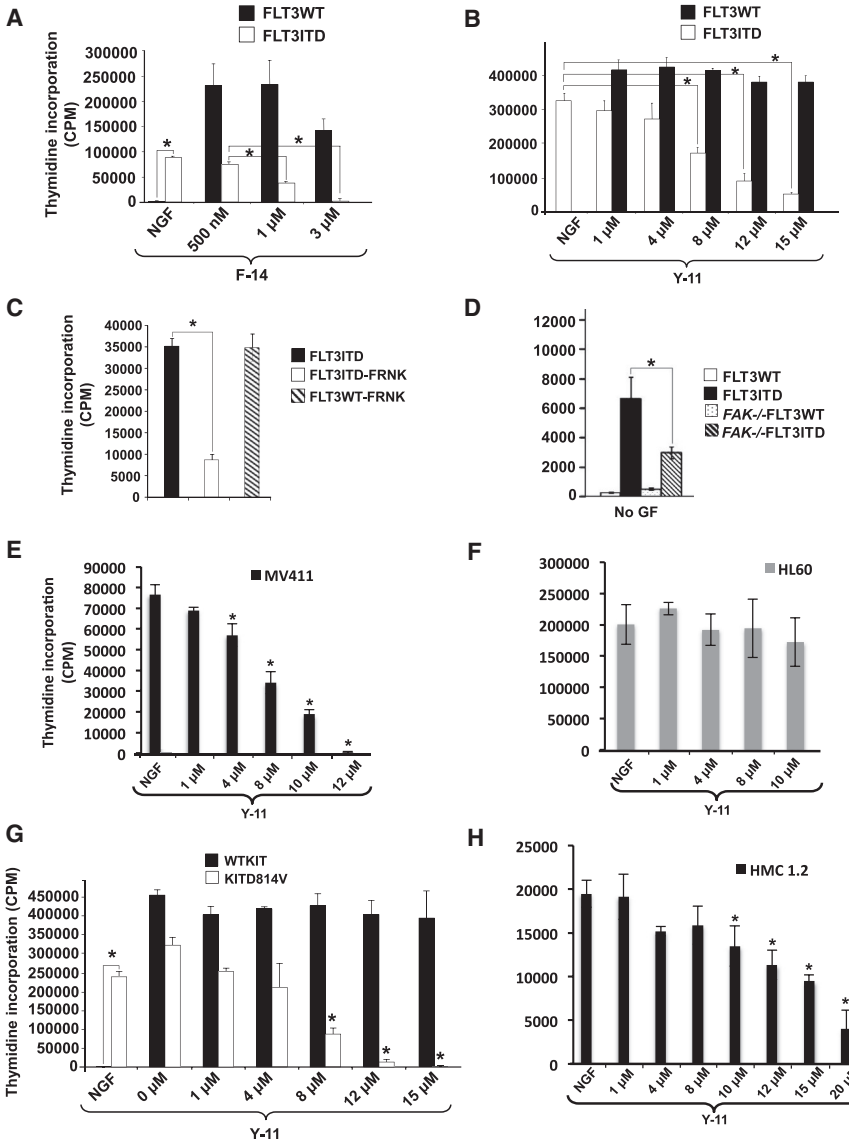

Figure 2. Inhibition of FAK Suppresses the Constitutive Growth of Oncogenic FLT3- and KIT-Bearing Cells

(A and B) BaF3 (A) or 32D (B) cells expressing FLT3WT or FLT3ITD were cultured for 48 hr in the presence or absence of F-14 or Y-11 in replicates of four and subjected to a thymidine incorporation assay. CPM, counts per minute.

(C) BaF3 cells coexpressing FRNK and either FLT3ITD or FLT3WT were subjected to thymidine incorporation assay as in (A) and (B).

(D) WT or *FAK*−/− BM cells expressing FLT3ITD or FLT3WT were subjected to proliferation assay in the absence of growth factors as described in (A) and (B).

(E and F) MV4-11 cells expressing endogenous levels of FLT3ITD (E) or HL60 cells harboring FLT3WT (F) were subjected to thymidine incorporation assay in presence of Y-11.

(G and H) 32D cells expressing KITD814V or WTKIT (G) and HMC1.2 human leukemic cells line harboring KIT (D816V + G560V) mutations (H) were cultured in the absence or presence of Y-11 and subjected to thymidine incorporation assay.

Thymidine incorporation is depicted on y axis as mean ± SD; *p < 0.05. NGF/No GF, cells grown in presence of no growth factors/cytokines. Data are representative of at least three independent experiments.

Y-11; another FAK-specific inhibitor (Golubovskaya et al., 2012) on FLT3ITD mediated repression of FAK. As seen in Figure 1H, the percentage of cells showing activated FAK was significantly higher in FLT3ITD-expressing cells (83.1%, middle panel) as compared to WT (19.2%; left versus middle panel). Treatment of FLT3ITD cells with Y-11 inhibited the activation of FAK (69.7%, right panel) and correspondingly increased the levels of unphosphorylated FAK (30.3%) in comparison to vehicle treated (16.9%; right panel versus middle panel). These results suggest that FAK is hyperactive in FLT3 and KIT oncogene-bearing cells and pharmacologic inhibition or genetic loss of FAK can repress the activation of FAK in these cells.

Inhibition of FAK Suppresses the Constitutive Growth of Oncogenic FLT3- and KIT-Expressing Cells

We assessed the functional significance of constitutive activation of FAK in FLT3ITD-expressing cells. As seen in Figure 2A, treatment of BaF3 cells with F-14 significantly repressed the ligand-independent growth of FLT3ITD-bearing cells in a dose-dependent manner, with minimal effect on FLT3WT-expressing cells. Similar growth repression was seen in 32D cells treated with Y-11 (Figure 2B). Expression of FRNK, a dominant negative version of FAK (Zhao and Guan, 2009), also repressed the ligand-independent growth of FLT3ITD-expressing cells (Figure 2C), which was partly a result of reduced survival (Figures S1A and S1B). Expression of FRNK in FLT3 cells is shown in Figure S8D. Although these results suggest an essential role for FAK in ligand-independent growth of FLT3ITD-expressing cells, a direct role of FAK was ascertained by complete ablation of FAK. As seen in Figure 2D, expression of FLT3ITD in WT BM cells (*FAK*+/+) demonstrated ligand-independent growth, which was significantly repressed in *FAK*−/− cells. We also assessed whether targeting hyperactive FAK in cell lines derived from human leukemic patients shows similar effects. We used MV4-11 and HL60 cells that express the FLT3ITD and WT receptors, respectively. Treatment of MV4-11 cells with Y-11 showed a dose-dependent repression of constitutive growth (Figure 2E), whereas no such effect was observed in HL60 cells (Figure 2F).

To assess whether FAK plays a similar role in cells bearing an oncogenic form of KIT (KITD816V in humans and KITD814V in mouse), we used 32D cells expressing WTKIT or KITD814V. As seen in Figure 2G, treatment of these cells with Y-11 resulted

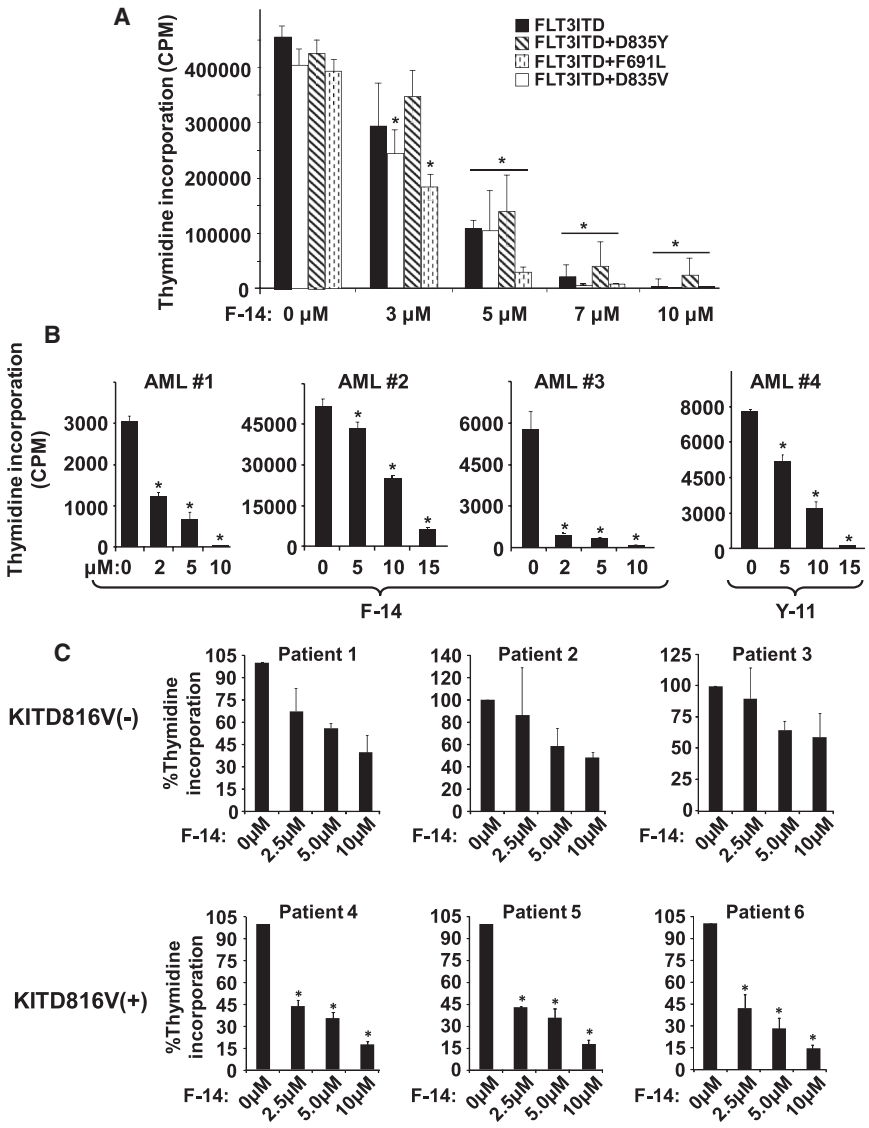

Figure 3. AC220-Resistant FLT3 Mutations, Primary AML FLT3ITD+ Cells, or KITD816V+ SM Cells Are Sensitive to FAK Inhibition

(A) BaF3 cells bearing FLT3ITD or FLT3 receptors with acquired AC220-resistant mutations in the kinase domain (D835Y, F691L, and D835V) were subjected to proliferation assay as described in Figure 2; *p < 0.05.

(B and C) Primary AML patient cells positive for FLT3ITD mutation (AML nos. 1–4) (B) or primary KITD816V(+) or KITD816V(−) SM cells (C) were treated with indicated concentrations of F-14 or Y-11. After 48 hr, proliferation assay was performed. Bars denote mean ± SD; *p < 0.05.

in growth repression of KITD814V-expressing cells. Similar results were observed upon Y-11 treatment of HMC1.2 cells derived from a human mastocytosis patient bearing the activating KIT mutation (Figure 2H). These results show that FAK plays an essential role in supporting the constitutive growth of FLT3 and KIT oncogene-bearing hematopoietic cells, which is modulated by pharmacologic or genetic inhibition of FAK.

AC220-Resistant "Driver" Mutations of FLT3 Are Sensitive to FAK Inhibition

Recent translational studies validated FLT3ITD mutations in AML to function as "driver," but not "passenger," mutations (Smith et al., 2012). Smith et al. demonstrated the presence of point mutations at three residues within the kinase domain of FLT3ITD that conferred resistance to AC220, an inhibitor of FLT3 and KIT. Acquisition of AC220-resistant substitutions at two of these residues was observed in all FLT3ITD+ AML patients with acquired resistance to AC220, thus validating FLT3ITD to function as a driver mutation and a critical therapeutic target in AML. We assessed whether these mutants were sensitive to FAK inhibition. As seen in Figure 3A, AC220-resistant FLT3-kinase-domain-mutant (D835Y, F691L, and D835V)-induced growth is inhibited by F-14.

AML FLT3ITD+ and KITD816V+ SM-Patient-Derived Cells Are Sensitive to FAK Inhibition

Next, we assessed whether inhibition of FAK in primary FLT3ITD+ AML cells inhibits their growth. We examined cells derived from 16 independent patients. Data from four representative patients are shown. In Figure 3B, a dose-dependent reduction in the growth of all FLT3ITD+ AML cells was observed in the presence of F-14 and Y-11. Likewise, SM-patient-derived cells positive for KITD816V mutation also demonstrated significantly greater growth reduction relative to patients lacking the expression of KITD816V (Figure 3C). These results demonstrate that FAK is indeed hyperactive in FLT3ITD- and KITD816V-expressing cells and its inhibition is associated with enhanced apoptosis and growth repression.

FAK and Rac1 Modulate the Nuclear Translocation of Active Stat5 in FLT3 and KIT Oncogene-Expressing Cells

In an effort to identify downstream targets of FAK that might contribute to FLT3ITD-induced growth and enhanced survival, we examined the activation of phosphatidylinositol 3-kinase (PI3K), ERK mitogen-activated protein kinase (MAPK) as well as Rac guanosine triphosphatases (GTPases). In nonhematopoietic cell types, all three pathways have been shown to be regulated by FAK (Gabarra-Niecko et al., 2003; Yin, 2011). We found constitutive and enhanced activation of Rac1 in FLT3ITD-bearing cells relative to FLT3WT-bearing cells (Figure 4A; lanes 1 and 2 versus 4 in control [CT] panel). Expression of FRNK in FLT3ITD-expressing cells significantly inhibited the

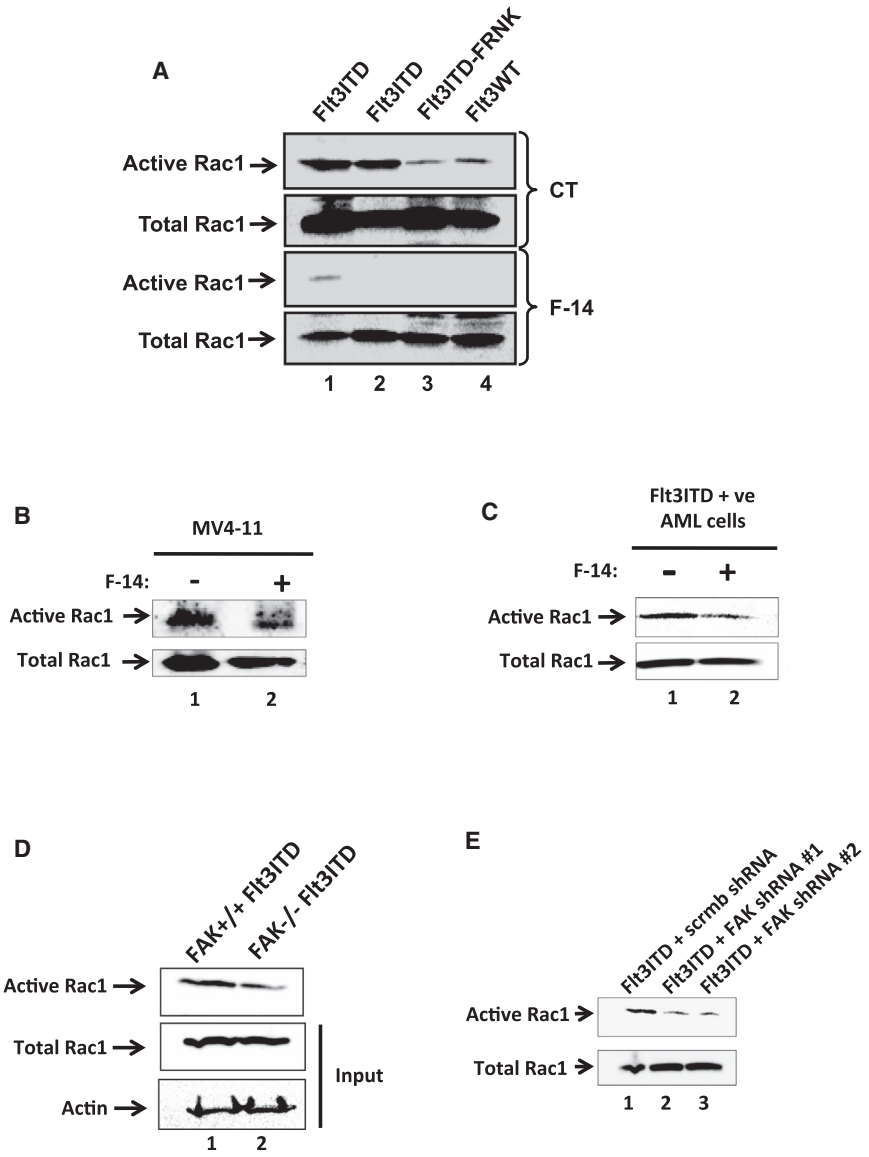

Figure 4. Rac1 Is a Downstream Effector of FAK in FLT3ITD-Bearing Oncogenic Pathway

(A) BaF3 (lane 1) or 32D (lane 2) cells expressing the FLT3WT receptor or FLT3ITD both alone or in combination with FRNK were starved and subjected to a Rac activation assay. These cells were either vehicle-treated alone (upper panel [CT]) or with F-14 (lower panel; n = 2).

(B and C) MV4-11 cells expressing endogenous FLT3ITD (B) and AML patient FLT3ITD+ cells (C) were subjected to Rac activation assay as described in (A).

(D) FLT3ITD-bearing BM cells in the setting of FAK deficiency were subjected to Rac activation assay as in (A) (n = 2).

(E) 32D FLT3ITD cells expressing shRNAs targeting FAK (lanes 2 and 3) and control shRNA (lane 1) were subjected to Rac1 activation assay as described in (A).

constitutive activation of Rac (Figure 4A; lane 3, CT panel). Furthermore, treatment of these cells with F-14 abolished Rac activation (lanes 1–4 upper CT panel versus lanes 1–4 lower [F-14] panel; Figure 4A). A similar reduction in Rac activation was also observed in FLT3ITD+ve human leukemic MV4-11 cells and FLT3ITD+ AML-patient-derived cells, respectively (Figures 4B and 4C) as well as in *FAK−/−*-deficient cells expressing FLT3ITD (Figure 4D). Small hairpin RNA (shRNA)-mediated downregulation of FAK in FLT3ITD-bearing cells also showed similar results (Figure 4E). These results suggest that FAK plays a role in the activation of Rac1 in FLT3ITD-bearing cells. Consistent with these observations, treatment of cells with a Rac1 inhibitor NSC23766 repressed the constitutive growth of FLT3ITD+ AML-patient-derived cells and of MV4-11 leukemic cells expressing the FLT3ITD receptor (Figures S1C and S1D). HL60 cells that express the FLT3WT receptor were used as a negative control (Figure S1E). Similar findings were observed in FLT3ITD-bearing cells expressing a dominant-negative version of Rac (RacN17; Figure S1F).

Active Stat5 is thought to play an essential role in regulating the transformation of FLT3 and KIT oncogene-bearing cells (Baumgartner et al., 2009; Brady et al., 2012). Although Stat5 is a transcription factor, mechanism(s) involved in the transport of this molecule in and out of the nucleus in oncogene-bearing cells remains poorly understood. We therefore performed cellular fractionation assays to determine the mechanism(s) behind active Stat5 translocation into the nucleus mediated by Rac1 and its upstream activator FAK in oncogene-bearing cells. We found active Stat5 in the nuclear fractions of FLT3ITD-bearing cells, which was associated with enhanced presence of Rac1, as compared with cells expressing FLT3WT (Figure 5A, lane 1 versus 3). To determine whether nuclear translocation of Stat5 could be mimicked in FLT3WT-expressing cells upon cytokine stimulation, cells were treated with IL-3 and analyzed for Stat5 and Rac1 localization. As seen in Figure 5A (lane 2), addition of IL-3 resulted in activation of FLT3WT-mediated Stat5-Rac1 nuclear localization. Next, we assessed if the effect of FAK inhibition could be overcome after ligand stimulation. FLT3ITD-expressing cells were treated with FLT3 ligand, followed by treatment with F-14. As seen in Figure 5B, stimulation of FLT3ITD with its ligand FL resulted in a modest increase in nuclear localized active Stat5 and Rac1 (Figure 5B, lane 1 versus 3; Zheng et al., 2011), which was repressed in the presence of F-14 (Figure 5B, lane 4). A similar reduction in the activation of Stat5 and accumulation of Rac1, respectively, was noted in the nuclear fractions of primary FLT3ITD+ AML (Figure S2A), KITD816V+ mastocytosis (HMC1.2; Figure 5C), and AML-patient-derived cells (MV4-11)

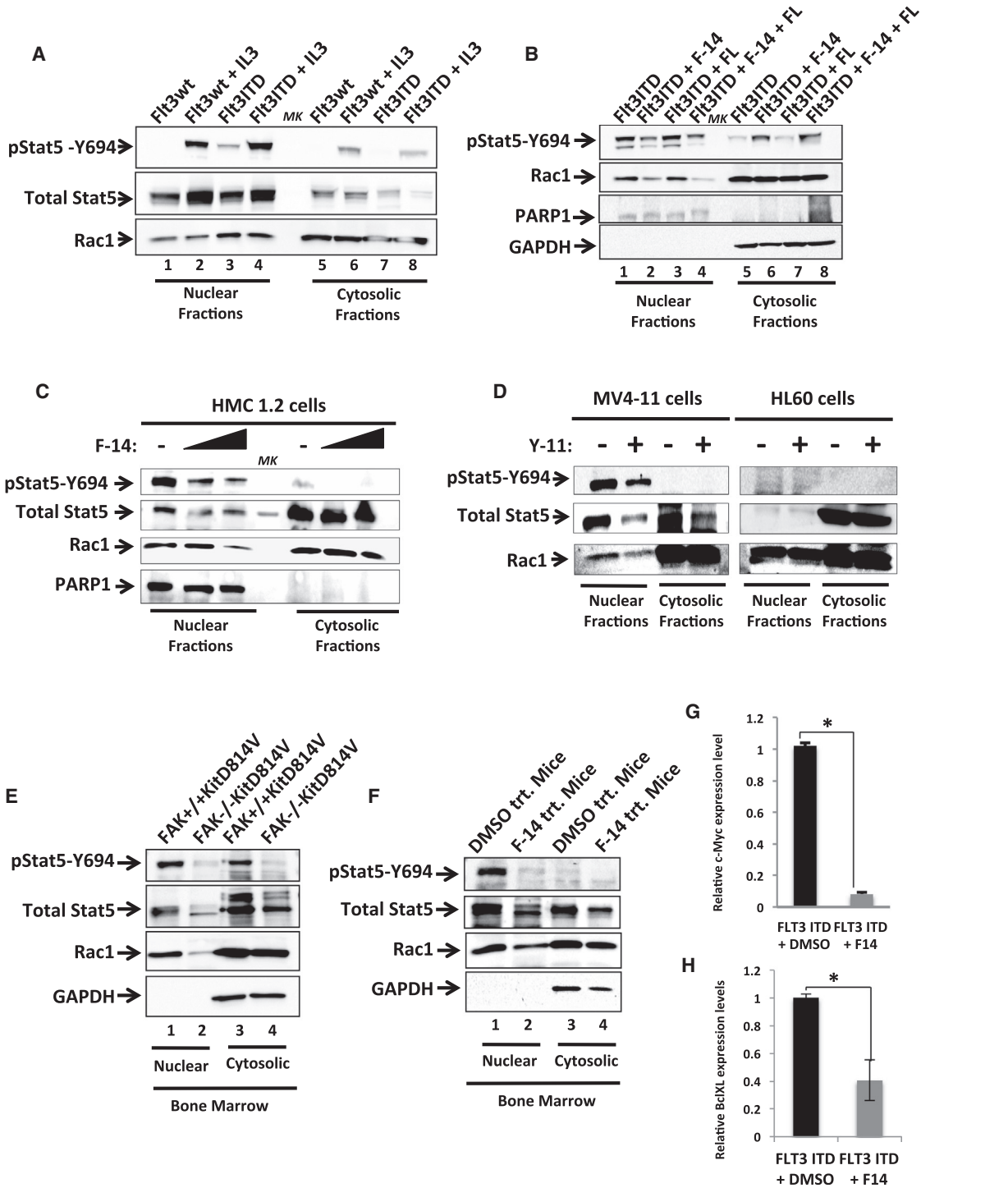

Figure 5. FAK Regulates the Translocation of Active Stat5 and Rac1 to the Nucleus in FLT3ITD- and KITD816V-Expressing Cells

(A) 32D cells expressing FLT3ITD or FLT3WT were serum starved and subjected to fractionation assays and nuclear and cytosolic fractions analyzed for levels of active Stat5 (pY694), total Stat5, and Rac1.

(B) 32D cells expressing FLT3ITD were subjected to fractionation assays after treatment with DMSO control, F-14, FL, or with F-14 followed by FL (B; n = 3).

(C) HMC1.2 cells bearing KITD816V+G560V mutations were subjected to fractionation assay as described in (A).

(D) MV4-11 and HL60 cells derived from leukemia patients harboring endogenous FLT3ITD and FLT3WT mutations were subjected to fractionation assay in presence of F-14 or Y-11 as in (A).

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treated with F-14 (Figure 5D). HL60 cells that express the FLT3WT receptor served as a negative control (Figure 5D, right panels). AC220-resistant driver mutations of FLT3 also demonstrated reduced nuclear accumulation of active Stat5 and total Rac1, respectively, when treated with F-14 (Figure S2B). Although we did not observe inhibition in the activation of MAPK or PI3K/Akt pathway in FLT3ITD-bearing cells lacking FAK or in which FAK was inhibited, we nonetheless directly examined the contribution of these pathways in the nuclear translocation of Stat5 and total Rac1. We performed a fractionation assay in the presence of MEK (PD98059), Raf (PLX4720), or Akt (124005) inhibitor and observed no significant inhibition of Rac1 or active Stat5 nuclear translocation (Figure S2C). Raf and MEK inhibitors also failed to repress the constitutive growth of FLT3ITD-bearing cells (Figures S2D and S2E).

A direct role for FAK in regulating the translocation of active Stat5 can be seen in Figure S2F. Expression of FLT3ITD failed to translocate active Stat5 and total Rac1 to the nucleus in *FAK*−/−-deficient BM cells (Figure S2F, lane 1 versus 2). The expression of FLT3 receptor in WT (*FAK*+/+) and *FAK*−/− BM cells is shown in Figure S8E. To further understand the mechanism behind FAK-regulated Stat5 translocation in FLT3 and KIT oncogene-bearing cells in an in vivo setting, we used cells derived directly from mice that were transplanted with BM from WT (*FAK*+/+) and FAK-deficient (*FAK*−/−) mice expressing KITD814V. Mice transplanted with WTFAK BM cells expressing KITD814V came down with disease significantly earlier than *FAK*−/− KITD814V mice (Figure 7G), at which point time-point-matched control mice (*FAK*−/− KITD814V) were euthanized and BM cells harvested from both groups of mice and subjected to cellular fractionation analysis. As seen in Figure 5E (lane 1 versus 2), and consistent with our in vitro findings (Figure S2F), genetic ablation of FAK in vivo inhibited the nuclear translocation of active Stat5 and Rac1 in KITD814V-bearing BM-derived leukemic cells. Furthermore, similar results were also observed after F-14 treatment of leukemic mice transplanted with BM cells expressing KITD814V. As seen in Figure 5F, BM cells derived from leukemic mice bearing the KITD814V mutation that were treated with F-14 also showed a reduction in Stat5 activation and nuclear accumulation of Rac1, as compared to cells derived from vehicle (DMSO)-treated mice (Figure 5F, lane 1 versus 2).

Active Stat5 downstream of FLT3ITD translocates to the nucleus to bind DNA and express Stat5-responsive genes like c-Myc and BclXL that play a crucial role in leukemogenesis (Li et al., 2007; Zhang et al., 2000). We next performed quantitative RT-PCR (qRT-PCR) analysis to determine the relative expression of c-Myc and BclXL genes in FLT3ITD cells treated with F-14. As seen in Figures 5G and 5H, a significant reduction in the expression of Stat5-responsive genes c-Myc and BclXL was observed upon FAK inhibition. Similar results were observed in *FAK*−/− FLT3ITD cells (Figures S3A and S3B). To ascertain whether FAK also regulates the nuclear association between Rac1 and Stat5, besides directly activating Rac1, F-14-treated nuclear fractions were subjected to Rac1 immunoprecipitation assay. As seen in Figure 6A, the amount of active Stat5 that interacts with Rac1 was significantly reduced upon treatment of FLT3ITD cells with F-14 (Figure 6A, lane 1 versus 2). These results explain our initial observation demonstrating reduced levels of total Rac1 and active Stat5 in nuclear fractions of FLT3ITD-bearing cells in which FAK activation was repressed and is directly a consequence of FAK's role in the activation of Rac1 and more importantly its association and subsequent translocation with active Stat5 into the nucleus. To further assess whether FAK regulates the association between active Rac1 and active Stat5, we performed active Rac1 pull-down assay from WT and *FAK*−/− BM cells expressing FLT3ITD and analyzed for active Stat5 binding. As seen in Figure 6B, increased levels of active and total Stat5 protein were bound to activated Rac1 in WT (*FAK*+/+) BM cells (lane 1), which was reduced in FAK-deficient (*FAK*−/−) FLT3ITD-bearing cells (lane 2). Taken together, these findings suggest that first FAK regulates the formation of an active Rac1-active Stat5 complex and second it modulates the translocation of Rac1-Stat5 complex into the nuclear compartment.

Because Rac1 contains a functional nuclear localization signal and also forms a complex with active Stat5 in the nucleus, we next investigated the role of Rac1 in nuclear translocation of active Stat5 in FLT3ITD-bearing cells. Cells were treated with or without Rac inhibitor NSC23766 and subjected to a fractionation assay. Levels of active Stat5, along with total Rac1, were reduced in FLT3ITD cells treated with NSC23766 (Figure S3C). Likewise, cells coexpressing FLT3ITD along with a dominant-negative form of Rac1 (Rac1N17) showed reduced levels of active Stat5 in the nuclear fractions (Figure S3D). Equal expression levels of GFP-Rac1N17 in FLT3ITD and WT cells can be seen in Figures S3E and S3F. As seen in Figure 6C (lane 1 versus 2), robust levels of active Stat5 were present in nuclear fractions of FLT3ITD-expressing *Rac1*+/+ BM cells, whereas a significant reduction in the levels of nuclear localized active Stat5 was observed in *Rac1*−/− BM cells, with no detectable levels of Stat5 activation in FLT3WT-bearing cells. These results demonstrate an essential role for Rac1 in the translocation of active Stat5 into the nucleus.

RacGEF Tiam1 Is Essential for FLT3ITD-Induced Leukemic Development in Mice

To identify Rac guanine nucleotide exchange factors (GEFs) involved downstream of FAK in activating Rac1 in FLT3ITD-bearing cells, we analyzed the role of Tiam1. We ascertained whether Tiam1 is active in cells bearing FLT3ITD. As seen in Figure 6D, increased levels of active Tiam1 were observed in

(E) Fractionation assays were performed in BM cells harvested from primary transplanted mice cohorts transplanted with KITD814V in a wild-type FAK (*FAK*+/+) or FAK-deficient (*FAK*−/−) background (n = 2).

(F) Fractionation assay from BM cells harvested from F-14- or DMSO (vehicle)-treated primary transplant mice cohorts. The level of Stat5 phosphorylation/expression and Rac1 expression in the nuclear and cytosolic fractions is indicated. Expression of GAPDH was used as an indicator of cytosolic marker and loading control. MK denotes lane with protein ladder.

(G and H) qRT-PCR analysis of relative mRNA expression levels of Stat5 responsive genes c-Myc (G) and BclXL (H) in FLT3ITD cells treated with F-14 or vehicle (DMSO; n = 2); *$p < 0.05$.

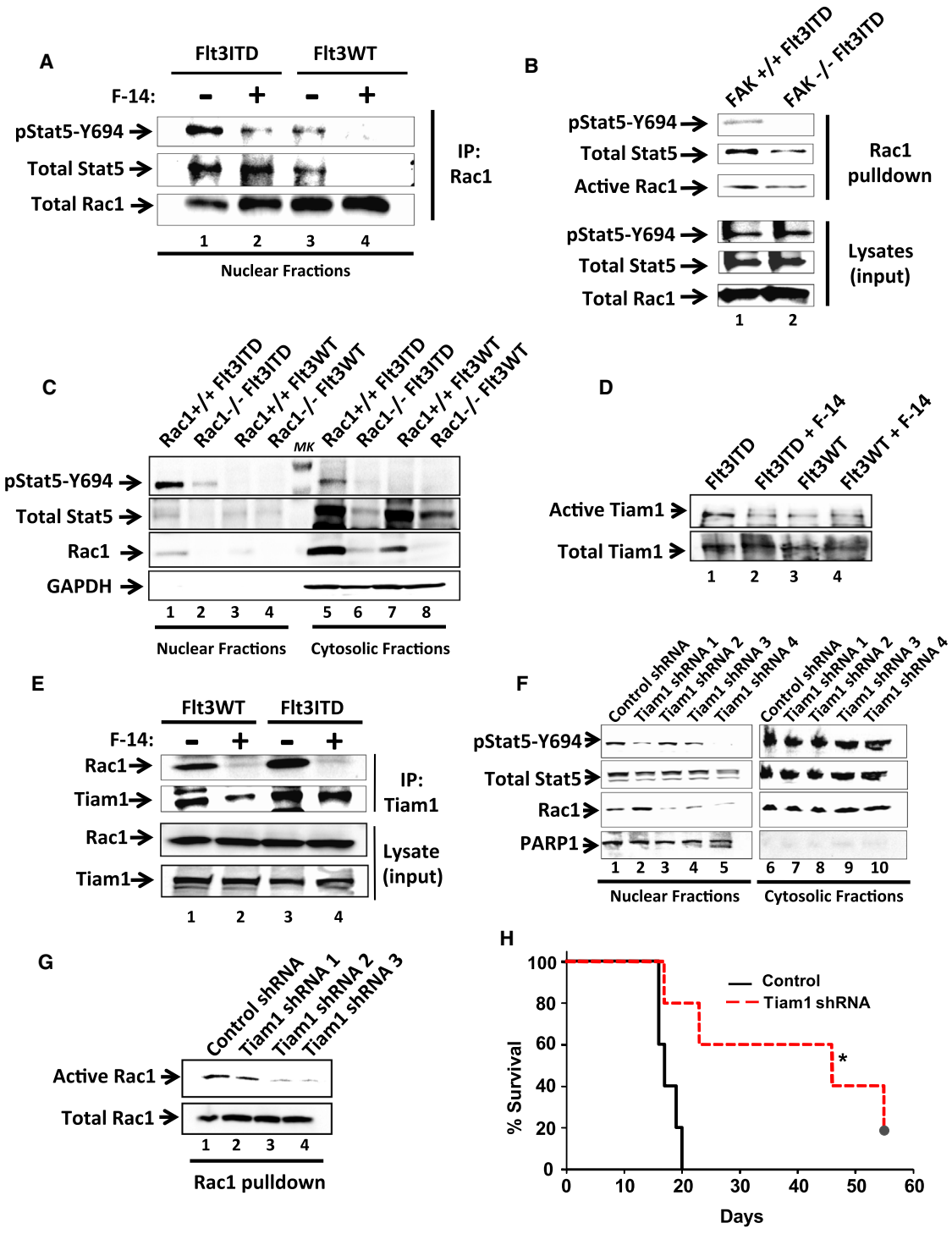

Figure 6. Downstream of FAK, Tiam1 Regulates the Activation of Rac1 and Subsequent Translocation of Active Stat5 to the Nuclear Compartment to Develop Leukemia in Mice

(A) Nuclear fractions from F-14-treated FLT3ITD- and FLT3WT-bearing cells were subjected to Rac1 immunoprecipitation assay to assess the level of Rac1 binding to active Stat5. Level of active Stat5 (pY694) and total Stat5 and Rac1 were analyzed.

(B) Active Rac1 fractions from WT and *FAK*−/−-deficient BM cells expressing FLT3ITD were determined, along with levels of active and total Stat5. Total Rac1 levels are shown in the lowermost panel (n = 2).

(C) Fractionation assay was performed using WT and *Rac1*−/− BM cells expressing FLT3ITD or FLT3WT receptors. Nuclear and cytosolic fractions were analyzed as described above. GAPDH was used as a loading control and cytosolic marker (n = 3). MK denotes lane with protein ladder.

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FLT3ITD-bearing cells compared to FLT3WT-expressing cells (lane 1 versus 3), which was attenuated upon treating these cells with F-14 (lane 1 versus 2). Further, F-14 treatment perturbed the interaction between Rac1 and Tiam1 (Figure 6E). Having observed the presence of Tiam1 in active Rac1 complex in FLT3ITD-bearing cells and the perturbation of this association upon FAK inhibition, we next examined the functional significance of Tiam1 in FLT3ITD-induced transformation. We knocked down Tiam1 using shRNA in cells bearing FLT3ITD (Figure S3G). Cells bearing FLT3ITD and Tiam1 shRNA showed significant reduction in constitutive growth in comparison to cells bearing scrambled shRNA (Figure S3H). Moreover, when FLT3ITD and Tiam1shRNA coexpressing cells were subjected to a fractionation assay, the levels of active Stat5 and total Rac1 in nuclear fractions were significantly reduced compared to scrambled shRNA-expressing cells (Figure 6F, lane 1 versus 2–5). As shown in Figure 6G (lane 1 versus 2–4), knockdown of Tiam1 significantly inhibited the activation of Rac1 compared to cells coexpressing FLT3ITD and scrambled shRNA. These results indicate that Tiam1 plays an important role in the activation of Rac1 in FLT3ITD-expressing cells, which in turn regulates the nuclear translocation of the Rac1/Stat5 complex. To further investigate the role of Tiam1 in FLT3ITD-induced leukemogenesis, we performed transplantation experiments. Figure 6H shows that mice transplanted with FLT3ITD and Tiam1 shRNA survived significantly longer compared to mice transplanted with FLT3ITD and scrambled vector (*$p < 0.01$).

Inhibition of FAK Delays the Onset of FLT3ITD- and KITD814V-Induced MPN Development and Prolongs the Survival of Mice

We next examined the in vivo impact of FAK inhibition on FLT3ITD- and KITD814V-induced leukemogenesis. Although mice bearing FLT3ITD cells treated with DMSO died within 30 days posttransplant, mice treated with F-14 showed significantly prolonged survival (Figure 7A). F-14-treated mice showed significantly reduced spleen weight (Figures 7B and 7C) and demonstrated absence of lesions in lungs compared to DMSO-treated mice (Figures S4A and S4B). Histopathologic analysis showed leukemic infiltration of myeloid cells and destruction of alveolar architecture in lungs and of normal architecture in spleens of DMSO-treated mice but significant improvement in the F-14-treated mice (Figures S4C and S4D). Moreover, F-14-treated mice also showed reduced percentage of leukemic cells in tissues (peripheral blood and spleen) as determined by the presence of GFP-positive cells (Figures S4E and S4F), relative to vehicle-treated mice. Likewise, F-14 treatment of mice transplanted with cells expressing KITD814V survived significantly longer than vehicle-treated mice (Figure S5A; *$p < 0.01$). We next assessed these findings in mice transplanted with primary BM cells expressing KITD814V. One cohort of mice was treated with F-14 and the other with vehicle (DMSO). As seen in Figure 7D, white blood cell (WBC) counts remained constant over the entire duration of F-14 treatment in KITD814V-bearing mice (6 weeks), whereas KITD814V-bearing mice treated with vehicle demonstrated a steady rise in WBC counts over time, a hallmark of MPN development and progression. At the end of 6 weeks, all mice were euthanized and analyzed. As seen in Figures 7E and 7F, vehicle-treated mice demonstrated significant enlargement of spleen compared to F-14-treated mice. Collectively, these data support the observation that targeting FAK rescues the development of FLT3ITD- and KITD814V-induced MPN in vivo. To further investigate the role of FAK in FLT3ITD-induced MPN, we knocked down FAK expression using shRNA in cells bearing FLT3ITD and transplanted into mice as described in Figure 7A. Knockdown of FAK not only repressed the constitutive growth of FLT3ITD-bearing cells (Figure S5B), but more importantly, mice transplanted with cells coinfected with FLT3ITD and FAK shRNA survived significantly longer compared to mice transplanted with FLT3ITD and scrambled vector (Figure S5C; *$p < 0.025$). To rule out nonspecific effects of F-14 and diminished, but not absolute, effects of shRNA-mediated knockdown of FAK, we performed transplantation studies using primary BM cells from WT (*FAK*+/+) and *FAK*−/−-deficient mice expressing the oncogenic KITD814V receptor. As seen in Figure 7G, genetic ablation of FAK significantly prolonged the survival of leukemic mice (*FAK*−/− KITD814V) compared to controls (*FAK*+/+ KITD814V). Leukemic mice harboring the KITD814V oncogene in the *FAK*−/− background demonstrated reduced spleen size (Figures 7H and 7I) and WBC counts relative to controls (Figure S5D). To further ascertain whether targeting FAK in the context of KITD814V-induced MPN inhibits the growth of cells that give rise to leukemia, we performed secondary transplants using BM cells derived from the primary cohorts. As seen in Figure 7J, mice transplanted with BM cells from primary donor harboring KITD814V in a FAK-deficient background (*FAK*−/− KITD814V) survived significantly longer than mice transplanted with BM from WT background (*FAK*+/+ KITD814V). The prolonged survival of these mice correlated with reduced splenomegaly (Figures 7K and 7L). Importantly, loss of FAK in hematopoietic stem cells did not impair the engraftment or the self-renewal of these cells (Lu et al., 2012).

Targeting PAK1 Inhibits the Nuclear Translocation of Active Stat5

To determine the functional role of p21-activated kinase (PAK), a downstream effector of Rac1, in Stat5 regulation and leukemogenesis, we utilized a recently described allosteric PAK inhibitor, IPA-3 (Deacon et al., 2008). As seen in Figure 8A (lane 5 versus 7),

(D) 32D FLT3ITD and FLT3WT cells were treated with DMSO or with F-14 and subjected to Tiam1 activation assay (n = 2).
(E) 32D FLT3ITD and FLT3WT cells were subjected to Tiam1 IP in presence or absence of F-14. Samples were analyzed for amount of Rac1-binding Tiam1 (IP:Tiam1 panels). Lower two panels (lysate [input]) depict the total protein levels of Rac1 and Tiam1 (n = 2).
(F) 32D cells coexpressing FLT3ITD and Tiam1 shRNA or scrambled shRNA were subjected to cellular fractionation assay, and nuclear and cytosolic fractions were analyzed for the levels of active Stat5 (pY694), total Stat5 and Rac1, and nuclear marker/loading control PARP-1.
(G) 32D cells coexpressing FLT3ITD and Tiam1 shRNAs (lanes 2–4) or scrambled shRNA (lane 1) were subjected to active Rac1 pull-down assay. The amount of active and total Rac1 are shown in the upper and lower panels, respectively.
(H) Kaplan-Meier survival curve of mice transplanted with 32D cells coexpressing FLT3ITD and Tiam1 shRNA (n = 5) or scrambled shRNA (n = 5); *$p < 0.01$.

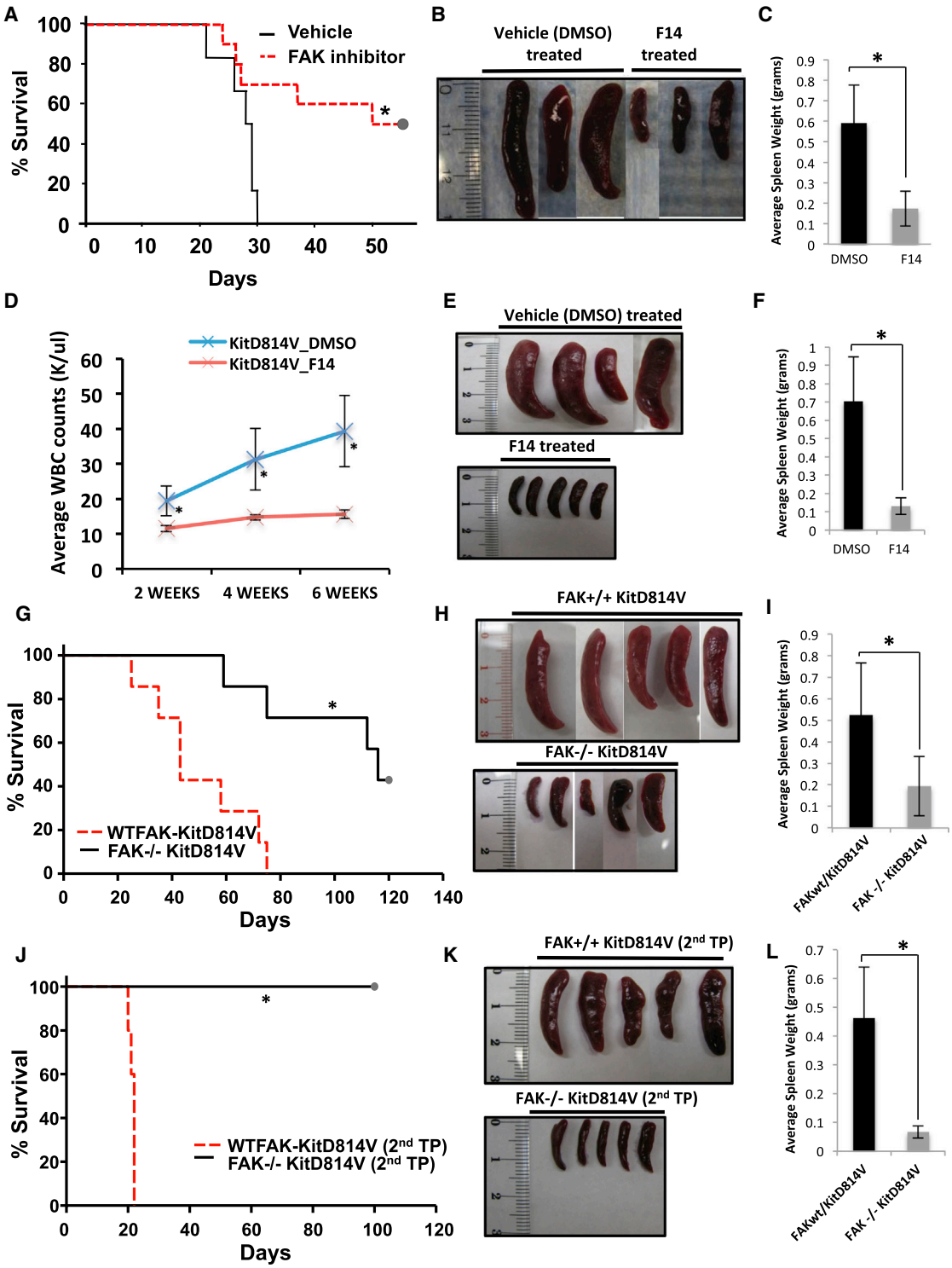

Figure 7. In Vivo Inhibition of FAK Delays the Development of MPN in Mice Transplanted with FLT3ITD- and KITD814V-Bearing Cells

(A) C3H/HeJ mice were transplanted with 32D cells bearing FLT3ITD and treated with 20 mg/kg body weight F-14 for 28 days.

(B and C) Kaplan-Meier survival analysis of vehicle- (n = 14) versus F-14 (n = 15)-treated mice showed significant increase in overall survival (*$p < 0.02$) and significant reduction of spleen size and weight in F-14-treated mice as compared to vehicle (DMSO) control-treated mice.

(D) BoyJ mice were irradiated and transplanted with 5′ fluorouracil (5-FU)-treated BM cells expressing KITD814V. Mice were randomly divided into two groups and treated with vehicle (DMSO; n = 7) or F-14 (n = 7) after 3 weeks posttransplantation, for 6 weeks. Peripheral blood from mice was analyzed at intervals of 2, 4, and 6 weeks.

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IPA-3 treatment significantly inhibited the presence of activated nuclear Stat5 in FLT3ITD-expressing cells. As seen in Figure 8A (lane 1 versus 2), addition of ligand also resulted in activation of the Stat5 oncogenic pathway in FLT3WT cells, which was also inhibited by IPA-3 (Figure 8A, lane 4). Next, we used another PAK inhibitor PF-3758309 (PF) (Murray et al., 2010). As seen in Figure 8B (lane 1 versus 2), similar results were observed upon treating FLT3ITD cells with PF-3758309, resulting in repression of nuclear translocation of active Stat5. Consistent with these results, expression of a dominant-negative form of PAK1 (K299R) in FLT3ITD-expressing cells (Figure S5F) demonstrated reduced tyrosine-phosphorylated Stat5 (Figure S5E). These results suggest that, downstream from Rac1, PAK contributes to the translocation of active Stat5 in the nucleus in FLT3ITD-bearing cells. To determine whether PAK is active in FLT3ITD and KITD814V oncogene-bearing cells, whole-cell lysates were analyzed for PAK1 activity. As seen in Figure S6A, cells expressing KITD814V showed increased levels of constitutive PAK1 activation (pPAK1) compared to KITWT cells (lane 1 versus 5), which was readily inhibited upon treatment with F-14 (lane 2 versus 6). Similar results were seen in FLT3ITD-expressing cells treated with F-14 (Figure S6B, lane 1 versus 2). Inhibition of active PAK1 levels was also observed in FLT3ITD-expressing cells treated with PAK inhibitors IPA-3 and PF-3758309 (Figure S6C, lane 1 versus 2 and 3). These results suggest that PAK1 is hyperactive downstream of a FAK/Rac1-signaling pathway in FLT3ITD and KITD814V oncogene-bearing cells.

Group I family of PAKs consist of three members including PAK1, PAK2, and PAK3. Whereas PAK1 and PAK2 are ubiquitously expressed, PAK3's expression is predominantly restricted to the brain (Ye and Field, 2012). We assessed the role of PAK1 and PAK2 in oncogene-induced transformation by examining Stat5 activation in FLT3ITD cells in which the expression of these two isoforms was knocked down (Figure S6E). As seen in Figure S6D, knockdown of PAK1 (lane 1 versus 2 and 3), but not PAK2 (lane 1 versus 4–6), significantly reduced the nuclear accumulation of active Stat5 in FLT3ITD-expressing cells. Consistent with these findings, expression of FLT3ITD in PAK1-deficient BM (*PAK1*−/−) cells also impaired the nuclear translocation of active Stat5 (Figure S6I, lane 1 versus 2). Furthermore, loss of active Stat5 nuclear import in *PAK1*−/− FLT3ITD cells resulted in reduced expression of Stat5 target genes including Bcl-xL and c-Myc compared to controls (Figure S6F) and also reduced relative mRNA levels of c-Myc and BclXL in FLT3ITD cells treated with PAK inhibitor PF-3758309 (Figures 8C and 8D).

Inhibition of PAK1 Delays the Onset of FLT3ITD- and KITD814V-Induced MPN and Prolongs the Survival of Mice

To determine the role of PAK isoforms in FLT3ITD- and KITD814V-mediated MPN development, BM transplant studies were performed. Transplantation studies utilizing myeloid cells bearing FLT3ITD in the context of PAK2 knockdown did not prolong the survival of leukemic mice compared to controls (Figure S6G). In contrast, PAK1 knockdown in the context of FLT3ITD expression significantly enhanced the lifespan of leukemic mice (Figure S6H). To further confirm PAK1's involvement in KITD814V-mediated leukemogenesis, we transplanted mice with KITD814V-bearing *PAK1*−/− BM cells or WT (*PAK1*+/+) controls. Mice transplanted with WTPAK1 BM cells expressing KITD814V came down with disease significantly earlier than *PAK1*−/− KITD814V mice (Figure 8G), at which juncture time-point-matched control mice (*PAK1*−/− KITD814V, *PAK1*+/+ KITWT, and *PAK1*−/− KITWT) were euthanized and BM (Figure 8E) and spleen (Figure 8F) cells harvested and subjected to cellular fractionation analysis. As seen in Figures 8E (lane 1 versus 2) and 8F (lane 1 versus 2), similar to our data with cell lines and PAK inhibitors, genetic ablation of PAK1 in vivo abrogated the nuclear translocation of active Stat5 in KITD814V-bearing BM-derived cells. As seen in Figure 8G, genetic ablation of PAK1 significantly prolonged the survival of leukemic mice (*PAK1*−/− KITD814V) compared to controls (*PAK1*+/+ KITD814V) and modulated the development of MPN in mice as shown by reduced splenomegaly and WBC counts (Figures 8H, S6J, and S6K). Mice transplanted with WTKIT did not demonstrate any signs of MPN and showed normal survival and spleen size (Figures 8H and S6J). To further ascertain whether targeting PAK1 in the context of KITD814V-induced MPN selectively impacts the leukemia initiating cell "LIC" population, we performed secondary transplants using BM cells from primary recipients. As seen in Figure 8I, mice transplanted with BM cells from primary donor harboring KITD814V in a PAK1-deficient background (*PAK1*−/− KITD814V) survived significantly longer than mice transplanted with BM from WT background (*PAK1*+/+ KITD814V). The survival of mice correlated with correction in spleen size (Figures 8I, 8J, and S6L), similar to primary transplants described above.

Inhibition of PAK Inhibits the Constitutive Growth of FLT3ITD+ AML Cells and KITD816V (+) SM-Patient-Derived Cells

To assess the functional consequence(s) of PAK1 repression on the growth and transforming ability of FLT3ITD- and KITD814V-bearing cells, we performed a proliferation assay using cells expressing FLT3 and KIT receptors treated with or without PF-3758309. A dose-dependent reduction in the growth of FLT3ITD- and KITD814V-bearing cells was observed, but not that of FLT3WT- and KITWT-bearing cells (Figures S7A, S7B, S7C, and S7D, respectively). Similar results were observed for FLT3ITD cells treated with the PAK inhibitor IPA-3 (Figure S7E). To further validate these observations, we performed similar studies in FLT3ITD-bearing cells coexpressing a

(E and F) After 6 weeks, mice were harvested to determine spleen size (E) and weight (F).
(G) 5-FU-treated BM cells from WTFAK or *FAK*−/− mice expressing KITD814V were transplanted into lethally irradiated C57BL/6 mice.
(H and I) Kaplan-Meier survival analysis of *FAK*+/+ KITD814V (n = 9) versus *FAK*−/− KITD814V (n = 9) mice; spleen size (H) and weight (I) is shown (*$p < 0.002$).
(J) Secondary transplants were performed using BM from *FAK*+/+ KITD814V and *FAK*−/− KITD814V primary recipients.
(K and L) Kaplan-Meier survival analysis of *FAK*+/+ KITD814V (n = 5) versus *FAK*−/− KITD814V (n = 5) is shown (*$p < 0.003$) and spleen size (K) and weight (L; *$p < 0.05$).

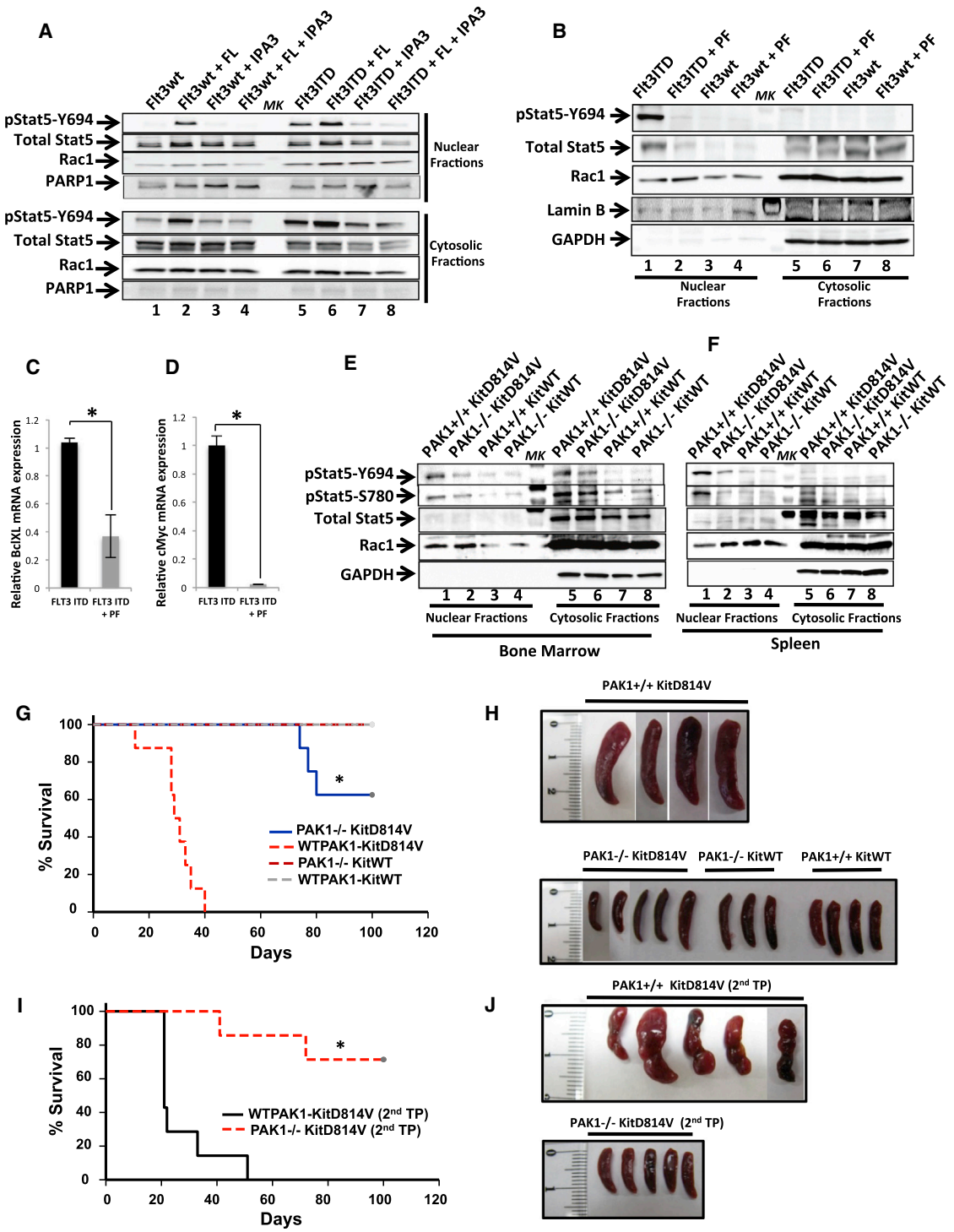

Figure 8. In Vivo Inhibition of PAK1 Delays the Onset of MPN in Mice Transplanted with KITD814V-Bearing Cells

(A) 32D cells expressing FLT3ITD or FLT3WT were starved of serum and treated with the PAK inhibitor IPA-3 (lanes 3 and 7) alone, with FL (lanes 2 and 6), or with IPA-3 followed by FL (lanes 4 and 8) as indicated and subjected to cellular fractionation assay. Nuclear and cytosolic fractions were quantitated, and equal lysates were loaded on a gel and probed with the indicated antibodies. Arrows indicate the activation/expression of the labeled molecules in nuclear as well as in cytosolic fractions of FLT3ITD- and FLT3WT-bearing cells. Expression of PARP-1 was used as an indicator of nuclear loading (n = 2).

(B) 32D FLT3ITD and FLT3WT were serum starved and treated with the PAK inhibitor PF-3758309 (PF) and analyzed as described in (A).

(legend continued on next page)

dominant-negative version of PAK1 (K299R; Figure S7F) as well as in BM cells expressing FLT3ITD in the setting of PAK1 deficiency (Figure S7G). A significant inhibition in the growth of FLT3ITD-bearing cells was noted in the background of dominant-negative or genetic ablation of PAK1. A similar inhibition in the growth of AML patient cells (Figure S7I) and KITD816V (+) SM-patient-derived cells (Figure S7K) was observed in the presence of IPA-3. Lastly, we assessed the role of PAK1 overexpression on the rescue of ligand-independent growth of BM cells expressing KITD814V in PAK1-deficient (*PAK1*−/−) background. An activated version of PAK1 (PAK1T423E; Schürmann et al., 2000) was coexpressed with KITD814V in BM cells derived from *PAK1*−/− mice. As seen in Figure S7H, we observed rescue of ligand-independent growth of cells overexpressing PAK1T423E, as compared to vector control.

DISCUSSION

Presence of active Stat5 in the nucleus and the subsequent expression of Stat5-dependent prosurvival and antiapoptotic genes plays a key role in the transformation of cells bearing oncogenic forms of FLT3 and KIT (Benekli et al., 2003; Choudhary et al., 2007; Tse et al., 2000). However, the mechanism(s) involved in regulating the active form of nuclear Stat5 remain poorly understood. To this end, a role for Rac1 GTPase/MgcRacGAP complex in the translocation of active Stat5 into the nucleus has been suggested; however, the upstream and downstream signaling proteins from Rac1 involved in this process have not been identified and the extent to which these proteins contribute to leukemogenesis is unknown (Sallmyr et al., 2008). Using pharmacologic, biochemical, and genetic approaches, we demonstrate that the FAK/Rac1-Tiam1/PAK1 axis plays a crucial role in the transformation induced by oncogenic forms of FLT3 (FLT3ITD) and KIT (KITD816V). Targeting FAK, Tiam1, and PAK1 in oncogene-bearing cells in vitro or in vivo inhibits the presence of active Stat5 in the nuclear compartment, which profoundly delays the onset of leukemia by repressing the expression of Stat5-responsive genes. These findings were validated in both murine and human models of AML and MPN and suggest that the signaling axis we have identified is highly conserved across species. More importantly, we show that this axis is active in leukemia-initiating cells as well as in leukemic cells that acquire AC220-resistant mutations of FLT3.

We and others have shown that FAK may contribute to Rac activation in hematopoietic cells as well as in other heterologous cell systems (Chang et al., 2007; Elias et al., 2010; Vemula et al., 2010) and that FLT3ITD can activate Rac1 and regulate the production of reactive oxygen species (ROS) via its association with Stat5 (Sallmyr et al., 2008). Furthermore, active Rac1 can also induce the activation and nuclear translocation of Stat3 (Simon et al., 2000). FLT3ITD can phosphorylate Stat5, independent of Jak kinase family members (Choudhary et al., 2007), whereas Rac1-mediated ROS production can induce the activation of Jak kinases and Stats downstream of G-protein-coupled receptors (Pelletier et al., 2003). Although significant work has been done in identifying the above described linkages, how these molecules connect and what is their relationship in regulating transformation via oncogenic forms of KIT and FLT3 has never been described. Our findings provide insight into how FAK-Rac1-Tiam1 and PAK1 axis contributes to leukemic transformation in part by regulating active nuclear Stat5.

The role of GEFs such as Vav1 and Vav2 is largely considered promiscuous, as they regulate the activity of all three members of the Rho GTPase family including Rac, Rho, and Cdc42 (Schmidt and Hall, 2002). In contrast, GEF Tiam1 is highly specific for Rac1 in vivo and has been implicated in activating Rac1 to mediate Stat3 activation and its subsequent nuclear localization in COS-1 cells (Simon et al., 2000). We demonstrate that FAK activates Rac1 via Rac GEF Tiam1 in FLT3ITD-bearing cells and targeting FAK and Tiam1 results in inhibition of Rac1. We also show that shRNA-mediated knockdown of Tiam1 prolongs the survival of FLT3ITD-bearing leukemic mice and genetic and pharmacologic inhibition of FAK and Tiam1 results in failure of active Stat5 to be expressed in the nucleus, along with Rac1. Whereas Tiam1 regulates epithelial cancers such as carcinomas of breast and colon (Bourguignon et al., 2000; Buongiorno et al., 2008), we show its role in regulating hematologic malignancies.

Although evidence in this study and reported earlier (Sallmyr et al., 2008) suggests a role of Rac1 in translocating Stat5 into the nucleus, the relationship between Stat5 and Rac GTPases in the context of FLT3 and KIT oncogenic mutations is unclear. An indirect role of PAK1 in nuclear shuttling of Stat5 has been suggested, where PAK1 plays a role in ''switching'' in occupancy of the same promoter region between BCL6 and Stat5. In colorectal cancer, PAK1, activated via Rac1, translocates into the nucleus and phosphorylates chromatin-bound BCL6, leading to its dissociation from the promoter, thereby allowing active Stat5 that is already present in the nucleus via a Rac1/MgcRacGAP-dependent mechanism to bind to the same promoter regions (Barros et al., 2012). In line with reported findings by Barros

(C and D) qRT-PCR analysis of relative mRNA expression levels of Stat5-responsive genes BclXL (C) and c-Myc (D) in FLT3ITD cells treated with PAK inhibitor PF-3758309 (n = 2); *p < 0.05.

(E and F) Fractionation assays were performed in BM cells (E) and splenocytes (F). Mice cohorts transplanted with KITD814V in a WT PAK1 (*PAK1*+/+ KIT814V) or PAK1-deficient (*PAK1*−/− KITD814V) cells (n = 2). The level of phospho-Stat5 and total Stat5 and Rac1 in the nuclear and cytosolic fractions is indicated. Expression of GAPDH was used as an indicator of cytosolic marker and loading control. MK denotes lane with protein ladder.

(G and H) Primary transplants were carried out using 5-FU-treated BM cells from WTPAK1 or *PAK1*−/− mice transduced with KITD814V or KITWT and transplanted into lethally irradiated C57BL/6 mice. Four groups of mice were used: WTPAK1 KITD814V (n = 8); WTPAK1 KITWT (n = 5); *PAK1*−/− KITD814V (n = 8); and *PAK1*−/− KITWT (n = 5). Kaplan-Meier survival analysis of *PAK1*+/+ KITD814V versus *PAK1*−/− KITD814V, WTPAK1 KITWT, and *PAK1*−/− KITWT mice showed significant overall survival (*p < 0.0003; Hom-Sidak method) and significant reduction of spleen size (H).

(I and J) Secondary transplants were performed with BM cells from *PAK1*+/+ KITD814V and *PAK1*−/− KITD814V mice and transplanted into irradiated C57BL/6 mice. Kaplan-Meier survival analysis of *PAK1*+/+ KITD814V (n = 5) versus *PAK1*−/− KITD814V (n = 5) mice showed significant overall survival (*p < 0.001) (I) and significant reduction of spleen size (J).

et al., we have observed a reduction in BCL6 activation and its mRNA expression levels upon treatment of FLT3ITD cells with PAK1 inhibitor (Figures S8A and S8B). These data demonstrate that, in the FLT3 and KIT oncogenic pathway, the FAK/Tiam1/Rac1-signaling axis activates PAK1, which in turn inhibits the transcriptional repressor BCL6, while correspondingly activating Stat5 to mediate leukemic transformation.

In BCR-ABL-induced CML, majority of Stat5 is persistently active and retained in the cytoplasmic compartment, primarily via an association of active Stat5 with Gab2 and PI3K/Akt, subsequently leading to leukemogenesis (Nyga et al., 2005). FLT3ITD also interacts with Gab2 and results in the activation of PI3K/Akt-; Stat5- and Gab2-mediated recruitment of Src kinases can also result in the activation of Stat5. Thus, whereas FLT3ITD can directly activate Stat5 (Choudhary et al., 2007), other tyrosine kinases present in complexes with FLT3ITD may also be involved in regulating Stat5 activation including Src kinases, as mutating Src-kinase-binding sites Y589 and Y591 in FLT3 receptor inhibits Stat5 activation (Hayakawa et al., 2000; Rocnik et al., 2006). Our data using FLT3ITD specific inhibitor AC220 not only show inhibition in the phosphorylation of FLT3ITD on Y589/591 but also downstream inhibition of activating residue Y397 on FAK, indicating that oncogenic FLT3 mediates direct activation of FAK. In breast cancer cells, prolactin (PRL)-induced activation of FAK and Stat5 is mediated via Src family kinases. Upon phosphorylation by Src, phosphorylated FAK recruits Grb2/Gab2 to mediate activation of Ras/MAPK-signaling pathway. The involvement of the PI3K/Rac/Pak pathway in PRL-induced activation of Erk has also been suggested. These results demonstrate a complex crosstalk between various signaling pathways involved in breast cancer metastasis (Aksamitiene et al., 2011). Future studies will determine whether Gab2 or other Src kinases play a role in activating FAK downstream of FLT3ITD and KITD814V receptors, which results in oncogenic transformation mediated by the subsequent nuclear translocation of active Stat5.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents

Phycoerythrin-conjugated annexin V antibody and 7-amino actinomycin D were purchased from BD Biosciences PharMingen. Rabbit anti-phospho-PAK1, anti-PAK1, anti-phospho-Stat5 (Y694), anti-Stat5 antibodies, rabbit anti-phospho-FAK (Y397), and anti-FAK antibodies were purchased from Cell Signaling Technology. Anti-rabbit immunoglobulin G DyLight 649 from Biolegend, Anti-actin and GAPDH antibodies were purchased from Sigma. Tiam1 activation kit was purchased from Cell BioLabs. Anti-mouse-horseradish peroxidase (HRP), anti-rabbit-HRP, anti-goat-HRP, anti-phospho-Stat5 (S780), anti-Tiam1, PARP-1, LaminB, BclXL, and cMyc antibodies were purchased from Santa Cruz Biotechnology. FAK inhibitors F-14 and Y-11, PAK inhibitors IPA-3 and PF-3758309, and Rac inhibitor NSC23766 were purchased from R&D Systems. Lumina Forte Western HRP Substrate, Chemiluminescent Blocker (bløk-CH), anti-phospho-FAK (Y397) rabbit polyclonal, anti-Rac1 (23A8), and Rac1 activation kit were purchased from Millipore. Recombinant murine and human IL-3, Flt3, granulocyte macrophage-colony stimulating factor, stem cell factor, IL-6, and Tpo were purchased from Peprotech. Retronectin was obtained from Takara. Iscove's modified Dulbecco's medium was purchased from Invitrogen. Monothioglycerol was purchased from Sigma. [^{3}H]thymidine was purchased from PerkinElmer. Protein A-Sepharose beads were purchased from Amersham Biosciences. MKK/MEK inhibitor PD98059 was purchased from Cell Signaling Technology, Raf inhibitor PLX4720 from Selleckchem, and Akt inhibitor 124005 from Millipore.

Mice

C57BL/6 and C3H/HeJ mice were purchased from Jackson Laboratory. FAK-, Rac1-, and PAK1-deficient mice have been previously described (Martin et al., 2013; McDaniel et al., 2008; Vemula et al., 2010). All mice used in this study were between 6 and 12 weeks of age and were maintained under specific pathogen-free conditions at the Indiana University Laboratory Animal Research Center. The studies were approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine.

Patient Samples

Peripheral blood mononuclear cells from patients with AML were obtained at the time of diagnostic testing after informed consent. Approval was obtained from the institutional review boards of Indiana University School of Medicine. The buoyant fraction was isolated over Ficoll-Hypaque and then washed with PBS before processing as described previously (Hartman et al., 2006). KITD816V-positive or negative SM-patient-derived cells were obtained as described (Traina et al., 2012).

Cells

Primary low-density mononuclear cells were harvested as described earlier and used in the study (Mali et al., 2011). The murine IL-3-dependent myeloid cell line 32D cells bearing FLT3, FLT3ITD (N51), MIEG3 vector, KIT, or KITD814V have been described (Mali et al., 2011). Puromycin-resistant BaF3 cells bearing the AC220-resistant mutants (Flt3ITD+TKD_D835Y/F and F691L) have been described (Smith et al., 2012). The human mast cell leukemia line, bearing the KITV560G as well as KITD816V mutations, HMC1.2 and AML cell line, bearing the FLT3ITD mutation, MV4-11 have been described (Butterfield et al., 1988; Lange et al., 1987).

Expression of WT and Oncogenic Receptors

Transduction of 32D and primary BM-derived hematopoietic stem and progenitor cells was performed as described previously (Mali et al., 2011).

shRNA Silencing of FAK, Tiam1, and PAK

FAK-, Tiam1-, or PAK-specific shRNA expression plasmids were purchased from OriGene Technologies. Purified and sequence-verified plasmid containing a noneffective 29 mer shGFP cassette (Scrambled vector) was used as a negative control. Cells were transduced with scrambled vector or shRNA plasmid and grown in the presence of puromycin (10 ng/ml) to select for the transduced cells.

Proliferation and Apoptosis Assays

Proliferation assays were performed as previously described (Mali et al., 2011).

Cytoplasm and Nuclear Extraction

To extract nuclear and cytosolic fractions, the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) was used as per the manufacturer's instructions.

IP and WB

Immunoprecipitation (IP) and western blot analysis (WB) was performed as described previously (Mali et al., 2011).

qRT-PCR

Total RNA was isolated from 5 × 10^6 cells using RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was generated using random hexamer primers and Superscript II reverse transcriptase (Invitrogen). qRT-PCR was performed using FastStart Universal SYBR Green master mix (Roche) and a Applied Biosystem 7500 Real Time PCR system. β-actin amplification was used to normalize sample RNA content.

Mouse Leukemia Induction and In Vivo Drug Treatment

Mouse leukemia induction and in vivo drug treatments were performed as described previously (Mali et al., 2011). We injected 1 × 10^6 32D cells bearing FLT3ITD in 200 μl PBS into C3H/HeJ mice intravenously. After 48 hr of transplantation, mice were treated with vehicle (PBS/DMSO) or FAK inhibitor F-14 (25 mg/kg body weight) by intraperitoneal injection at 24 hr interval for

21 days. Mice were closely monitored for MPN development and harvested at moribund. For F-14 treatment in a primary transplant model, irradiated BoyJ mice were transplanted with 2.0 × 10^6 GFP-KITD814V cells and 0.1 × 10^6 supporting BM cells. Three weeks posttransplant mice were treated with 10 mg/kg body weight F-14 for 5 days a week for 6 weeks. All mice were harvested, and peripheral blood (PB) counts were monitored using a Hemavet 950 (Drew Scientific). PB, BM, and spleens were analyzed for GFP expression. BM, spleen, and lungs were also fixed in 10% buffered formalin to perform histopathologic analysis by hematoxylin and eosin staining.

Primary and Secondary Bone Marrow Transplants

Eight- to ten-week-old C57BL/6, *PAK1*−/−, or *FAK*−/− mice were injected with 5-flurouracil (150 mg/kg body weight), and after 5 days, the mice were killed and BM was harvested. Cells were prestimulated overnight in Iscove modified Dulbecco medium supplemented with 20% fetal bovine serum with mouse SCF (100 ng/ml), mouse TPO (100 ng/ml), mouse Flt3 (50 ng/ml), and mouse IL-6 (10 ng/ml). Cells were plated on Retronectin-coated plates (Takara), and retroviral transduction was performed using viral supernatants as described before (Mali et al., 2011). GFP+ve cells were sorted using FACS Aria (BD Biosciences). We injected 1 × 10^6 GFP-positive cells along with 0.1 × 10^6 supporting splenocytes into the tail veins of lethally irradiated (11 Gy) female C57BL/6 recipient mice. Peripheral blood counts were monitored on a regular interval after transplantation on a Hemavet 950 (Drew Scientific) and by fluorescence-activated cell sorting analysis for GFP expression. All moribund mice were then euthanized, and peripheral blood, BM, and spleen cells were analyzed for GFP expression. At day 120, all remaining mice were euthanized and analyzed.

For secondary transplant, leukemic WT mice and time-point-matched *PAK1*−/− or *FAK*−/− mice, both bearing KitD814V-expressing cells, were harvested and 1 × 10^6 cells were injected into the tail veins of lethally irradiated (11 Gy) female C57BL6 recipients.

Statistics

All graphical data were evaluated by paired Student's t test (two-tailed), and results were considered significantly different with p value < 0.05. All data are represented as mean values ± SD. Survival probability of transplanted mice groups was compared using a Kaplan-Meier survival analysis in which statistical significance was determined as p values < 0.05 by log rank test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.039.

AUTHOR CONTRIBUTIONS

A.C. conceived, designed, performed, and analyzed experiments and wrote the manuscript. J.G. designed, performed, and analyzed experiments and edited the manuscript. B.R., R.S.M., H.M., M.K., S.V., V.H.C., and E.R.W. performed experiments. V.V., R.V.T., and C.C.S. provided reagents. N.S., K.D.B., H.S.B., Y.L., and R.J.C. provided expertise and reagents. R.K. conceived, designed, and analyzed experiments and wrote the manuscript.

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Bmi1 Promotes Erythroid Development Through Regulating Ribosome Biogenesis

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Key Words. Bmi1 • Ribosome biogenesis • Erythroid differentiation • p53 • Ribosomopathies

ABSTRACT

While Polycomb group protein Bmi1 is important for stem cell maintenance, its role in lineage commitment is largely unknown. We have identified Bmi1 as a novel regulator of erythroid development. Bmi1 is highly expressed in mouse erythroid progenitor cells and its deficiency impairs erythroid differentiation. BMI1 is also important for human erythroid development. Furthermore, we discovered that loss of Bmi1 in erythroid progenitor cells results in decreased transcription of multiple ribosomal protein genes and impaired ribosome biogenesis. Bmi1 deficiency stabilizes p53 protein, leading to upregulation of p21 expression and subsequent G0/G1 cell cycle arrest. Genetic inhibition of p53 activity rescues the erythroid defects seen in the Bmi1 null mice, demonstrating that a p53-dependent mechanism underlies the pathophysiology of the anemia. Mechanistically, Bmi1 is associated with multiple ribosomal protein genes and may positively regulate their expression in erythroid progenitor cells. Thus, Bmi1 promotes erythroid development, at least in part through regulating ribosome biogenesis. Ribosomopathies are human disorders of ribosome dysfunction, including Diamond-Blackfan anemia (DBA) and 5q− syndrome, in which genetic abnormalities cause impaired ribosome biogenesis, resulting in specific clinical phenotypes. We observed that *BMI1* expression in human hematopoietic stem and progenitor cells from patients with DBA is correlated with the expression of some ribosomal protein genes, suggesting that BMI1 deficiency may play a pathological role in DBA and other ribosomopathies. STEM CELLS *2015;33:925–938*

INTRODUCTION

Polycomb group proteins (PcGs) are epigenetic chromatin modifiers involved in stem cell maintenance and cancer development [1–3]. Biological and genetic studies indicate that PcG proteins exist in at least two separate protein complexes, Polycomb repressive complex 2 (PRC2) and Polycomb repressive complex 1 (PRC1), that act in concert to promote and maintain gene repression [2]. EZH2, the catalytically active component of PRC2, dimethylates and trimethylates Lys27 of histone H3, a histone mark recognized by the PRC1 complex through the chromodomain of Polycomb that triggers ubiquitination of histone H2A at Lys119 by the E3 ubiquitin ligase Ring1B in PRC1 [4–6]. Polycomb group proteins (PcGs) regulate lineage choices during development and differentiation through binding and repressing the promoters of hundreds of genes encoding proteins with roles in cell fate determination [1–3]. However, it is unclear how PcG proteins are displaced and recruited to different subsets of target genes during lineage commitment [3].

PcG proteins are key regulators of both hematopoietic stem cell (HSC) maintenance and terminal differentiation [7–9]. The PRC2 complex regulates normal hematopoietic stem cell function in a developmental-stage-specific manner [10]. While Ezh2 is important for fetal hematopoiesis [11], Ezh1 maintains the adult hematopoietic stem cell pool [12]. Polycomb group protein Bmi1 plays important roles in regulating hematopoietic stem cell self-renewal [13, 14] and we showed that Bmi1 is a critical down-stream target of Akt signaling and Akt-mediated phosphorylation of Bmi1 inhibits HSC self-renewal [15]. Bmi1 is also important for lymphoid development [16]. Epigenetic changes have been implicated in regulating the induction of erythroid-specific genes during terminal erythropoiesis [17]. Ezh2-deficient embryos died of anemia because of insufficient expansion of hematopoietic stem and progenitor cells (HSPCs) and defective erythropoiesis in fetal liver [11]. However, the role of Bmi1 in

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mammalian erythroid development is largely unknown.

Ribosome biogenesis is important for erythroid development and impaired ribosomal function has been implicated in ribosomopathies [18], including Diamond-Blackfan anemia (DBA), a congenital bone marrow (BM) failure syndrome [19], and the 5q− syndrome, a subtype of myelodysplastic syndrome (MDS) [20]. In these disorders, genetic lesions in ribosomal protein genes impair ribosome biogenesis, resulting in activation of the p53 tumor suppressor pathway, cell cycle arrest, and defective erythropoiesis [19, 21]. While the developing erythrocytes are highly sensitive to suboptimal levels of ribosomal proteins [19], how ribosome biogenesis is regulated in erythroid precursors remains elusive. In this study, we have identified a critical role of Bmi1 in regulating erythropoiesis and ribosome biogenesis. Bmi1 deficiency downregulates multiple ribosomal protein genes, activates the p53 pathway and blocks erythroid differentiation. In addition, we demonstrated that Bmi1 associates with the promoter of some ribosomal protein genes and enhances their expression in erythroid precursors. Furthermore, we observed that *BMI1* expression in human $CD34^+$ cells from patients with DBA correlate with the expression of some ribosomal protein genes, suggesting that BMI1 deficiency may play a pathological role in DBA and other ribosomopathies.

Materials and Methods

Mice

Bmi1-deficient mice were provided by Martin van Lohuizen (The Netherlands Cancer Institute, The Netherlands). The generation of $p53^{R248W}$ mice has been described previously [22]. Wild-type C57BL/6 (CD45.2) mice were purchased from the Jackson Laboratories (Bar Harbor, Maine, USA, http://www.jax.org/). All mice were maintained in the Indiana University Animal Facility according to IACUC-approved protocols, and kept in Thorensten units with filtered germ-free air.

Human DBA Patient Samples

BM samples were collected after informed consent from healthy donors and patients with DBA using a protocol approved by the Institute of Hematology & Hospital of Blood Diseases Ethics Committee at the Chinese Academy of Medical Sciences & Peking Union Medical College.

Colony-Forming Unit Assays

Mononuclear cells obtained from mouse BM were used for colony-forming unit (CFU)-E and burst-forming unit (BFU)-E assays. MethoCult 3234 (Stem Cell Technologies, Vancouver, Canada, http://www.stemcell.com) containing 3 U/ml rhEPO or containing 3 U/ml rhEPO, 20 ng/ml rmIL-3, and 50 ng/ml rmSCF (PeproTech, Rocky Hill, NJ, USA, http://www.peprotech.com) was used for CFU-E and BFU-E assays, respectively. CFU-E colonies were scored on day 3 and BFU-E colonies were scored on days 8–10. For BFU-E assay of human $CD34^+$ cells, infected cells were plated in MethoCult H4435 medium (Stem Cell Technologies, Vancouver, Canada, http://www.stemcell.com) and colonies were scored after 2 weeks.

Overexpression Assays

Retroviral vectors were produced by transfection of Phoenix E cells with the MIGR1 control or MIGR1 full-length Bmi1 c-DNA plasmids, according to standard protocols. Mouse hematopoietic progenitor cells were infected with high-titer retroviral suspensions in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA, https://www.sigmaaldrich.com). Twenty-four hours after infection, the green fluorescent protein (GFP)-positive cells were sorted by fluorescence-activated cell sorting (FACS).

Generation of Lentiviruses and Infection of Primary Hematopoietic $CD34^+$ Cells

Normal human cord blood (CB) samples were collected with institutional approval. Lentiviral vectors expressing short hairpins against human *BMI1* (CS-H1-shRNA-EF-1α-EGFP) and luciferase gene as a control were provided by Dr. Iwama at the Chiba University. Lentiviral particles were produced by transfection of 293T cells, according to standard protocols. After 24 hours of growth, $CD34^+$ cells were transduced on retronectin (Takara, Mountain View, CA, USA, http://www.clontech.com/takara)-coated nontissue culture plates with high-titer lentiviral concentrated suspensions in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA, https://www.sigmaaldrich.com). To induce erythroid differentiation, infected $CD34^+$ cells were maintained at 2×10^5/ml in StemSpan serum-free expansion medium (SFEM) containing erythropoietin (EPO) (6 IU/ml) and stem cell factor (SCF) (100 ng/ml) for 7 days. Then cells were harvested for flow cytometry and qPCR analysis.

Gene Expression and Pathways Analyses

Transcript profiling of Pro-E cells and megakaryocyte-erythrocyte progenitors (MEPs) from wild-type (WT) and $Bmi1^{-/-}$ mice were analyzed by Agilent Whole Mouse Genome Oligo Microarrays. Raw data will be available for download from Gene Expression Omnibus (http://ncbi.nlm.nih.gov/geo/, accession numbers GSE63411 and GSE63413). Genes whose expressions are increased or decreased more than twofold in $Bmi1^{-/-}$ cells compared to wild-type cells are shown. The microarray data were analyzed using the ingenuity pathways analysis program (Ingenuity Systems, Redwood City, CA, USA, www.ingenuity.com); to identify the pathways that met the less than or greater than twofold change cutoff and were associated with a canonical pathway in the ingenuity pathways knowledge base were considered for the analysis [23]. The significance of the association between the dataset and the identified canonical pathway was measured in two ways: (a) a ratio of the number of genes from the dataset that map to the pathway divided by the total number of genes from the dataset that map to the canonical pathway and (b) Fischer's exact test, to calculate a *p* value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Chromatin Immunoprecipitation Assays

For chromatin immunoprecipitation (ChIP) assays, K562 cells were fixed with 1% (vol/vol) formaldehyde for 10 minutes at room temperature. ChIP assays were performed using the EZ-Magna ChIP A/G Kit (Millipore, Billerica, MA, USA, http://www.emdmillipore.com). Anti-Bmi1 antibody (Active Motif, Carlsbad, CA, USA, http://www.activemotif.com; AF27), anti-Ring1b antibody (MBL International, Woburn, MA, USA, https://www.mblintl.com; D139-3), anti-H3K9Ac antibody (Millipore, Billerica, MA, USA, http://www.emdmillipore.com; 07-942), anti-

H3K4me3 antibody (Millipore, Billerica, MA, USA, http://www.emdmillipore.com; 07-473), anti-H3K27me3 antibody (Millipore, Billerica, MA, USA, http://www.emdmillipore.com; 07-449), anti-H2AK119ub1 antibody (Cell Signaling, Danvers, MA, USA, http://www.cellsignal.com; D27C4), and normal mouse IgG were used for immunoprecipitation. ChIP DNA was then subjected to real-time PCR analysis using primers targeting different region of ribosomal protein gene promoters [23, 24].

Polysome Analysis

Polysome analysis was performed as previous described [25, 26]. Equal numbers of control shRNA or BMI1 shRNA virus-infected K562 cells were used.

Statistical Analyses

Results from multiple experiments are expressed as the mean ± SD. Statistical significance (*, $p < .05$; **, $p < .01$; ***, $p < .001$; NS, not significant) was determined by Student's *t* test, one-way ANOVA, or two-way ANOVA.

Results

Bmi1 Regulates Mouse Erythroid Differentiation

We first assessed mouse *Bmi1* gene expression in purified hematopoietic stem and progenitor cells (LSKs), common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), megakaryocyte-erythrocyte progenitors (MEPs), proerythroblasts (Pro-E), basophilic erythroblasts (Baso-E), and chromatophilic erythroblasts (Chro-E) by quantitative real-time PCR. The LSKs, CMPs, and MEPs, as well as proerythroblasts (Pro-E), had high *Bmi1* expression. In contrast, GMPs and basophilic erythroblasts (Baso-E) had low *Bmi1* expression, whereas *Bmi1* transcripts were undetectable in the chromatophilic erythroblasts (Chro-E) committed to the erythroid lineage (Fig. 1A), indicating that Bmi1 may play a pivotal role in erythropoiesis. We next examined the peripheral blood of 10–12 weeks old *Bmi1* null mice and observed significantly decreased circulating red blood cell (RBC) in *Bmi1* null mice as compared to WT control. In addition, hemoglobin (Hb) concentrations were significantly decreased in *Bmi1* null mice. Peripheral blood RBCs in *Bmi1* null mice were larger than that in the WT mice, as assessed by mean corpuscular volume (MCV) (Fig. 1B).

Erythroid differentiation normally progresses from $CD71^{high}Ter119^{low}$ proerythroblasts (Pro-Es) via $CD71^{high}Ter119^{high}$ basophilic erythroblasts (Baso-Es), $CD71^{+}Ter119^{high}$ chromatophilic erythroblasts (Chro-Es), and $CD71^{-}Ter119^{high}$ orthochromatophilic erythroblasts (Ortho-Es) [27]. Analysis of erythroid development revealed ineffective erythropoiesis in the BM of $Bmi1^{-/-}$ mice characterized by a higher number of proerythroblast (Pro-E) and lower number of basophilic erythroblast (Baso-E), chromatophilic erythroblast (Chro-E), and orthochromatophilic erythroblast (Ortho-E) as compared to that of the wild-type mice (Fig. 1C), indicating that loss of Bmi1 impairs erythroid differentiation. We also found that *Bmi1* null BM cells formed strikingly less BFU-E and CFU-E colonies (Fig. 1D). To demonstrate that this effect is directly linked to the absence of Bmi1, we introduced Bmi1 into Bmi1 null BM cells using retroviruses that express Bmi1-IRES-GFP (MIGR1-Bmi1) or the empty IRES-GFP vector (MIGR1) and plated GFP-positive cells in methylcellulose medium. Introduction of Bmi1 increased both BFU-E and CFU-E colony formation of Bmi1 null cells compared with vector transduced cells (Fig. 1E).

It has been shown that Bmi1 null mice show defective HSC self-renewal and lymphoid differentiation [13]. To evaluate the effect of Bmi1 loss on erythromyeloid differentiation, we performed colony-forming unit (CFU) assays and observed that loss of Bmi1 decreased the colony formation of both granulocyte, erythrocyte, monocyte, and megakaryocyte (GEMM) and BFU-E (Supporting Information Fig. S1A), suggesting that more immature progenitors committed to the erythroid lineage may be affected. While the frequency of HSCs was dramatically decreased in Bmi1 null mice, the frequency of multipotent progenitors (MPPs) in the Bmi1 null BM was similar to that of WT mice [13]. We found that Bmi1 deficiency did not alter the frequency of CMP, GMP, and MEP (Supporting Information Fig. S1B). To determine the effect of Bmi1 deficiency on MEP function, we purified MEPs from wild-type and Bmi1 null BM cells and performed CFU assays. We found that loss of Bmi1 in MEPs decreased BFU-E colony formation (Supporting Information Fig. S1C), indicating MEPs are affected by Bmi1 deficiency. Collectively our data demonstrate that Bmi1 is essential for mouse erythropoiesis.

BMI1 Regulates Human Erythropoiesis

Despite the known role of human BMI1 protein in regulating human hematopoietic stem cell self-renewal [28, 29], the function of BMI1 in human erythropoiesis is largely unknown. To define the role of BMI1 in normal human erythropoiesis, we used RNA interference to reduce human *BMI1* gene expression in human CB $CD34^{+}$ HSPCs (Fig. 2A and Supporting Information Fig. S2) and then placed transduced cells in EPO (6 IU/ml)-driven liquid culture [30]. We monitored the generation of mature erythroid precursor cells (CD71/GlyA double positive cells) by flow cytometry and found that the generation of CD71/GlyA double positive cells was less efficient for the BMI1-knockdown (BMI-KD) cells, compared with the control shRNA-infected cells (Fig. 2B). To evaluate the sensitivity of BMI1-KD cells to EPO, we measured GlyA and CD71 expression by FACS analysis in the BMI1-KD HSPCs after plating these cells in culture with SCF (100 ng/ml) and different doses of EPO (0.5 and 6 U/ml). At all tested concentrations of EPO, the BMI1-KD cells showed delayed erythroid maturation compared with control cells (Fig. 2C and Supporting Information Fig. S3). We also found that knockdown of *BMI1* expression reduced the proliferation of $CD34^{+}$ cells in EPO-driven liquid culture (Fig. 2D) and decreased the formation of BFU-E colonies (Fig. 2E). Thus, BMI1 is important for the erythroid differentiation of human hematopoietic stem and progenitor cells.

Bmi1 Deficiency Results in Decreased Expression of Some Ribosomal Protein Genes in Erythroid Progenitor Cells

To investigate how Bmi1 regulates erythropoiesis, we performed transcript profiling assays to compare gene expression in MEPs isolated from wild-type and *Bmi1* null mice and observed that some genes important for erythropoiesis, including *Bcl11a*, *Hoxa10*, and *Stat5a*, are dysregulated in *Bmi1* null MEPs (Supporting Information Fig. S4A). We also performed transcript profiling assays to compare gene expression in proerythroblast cells ($CD71^{high}Ter119^{low}$ cells) isolated from wild-type and *Bmi1* null mice and used ingenuity pathways analysis

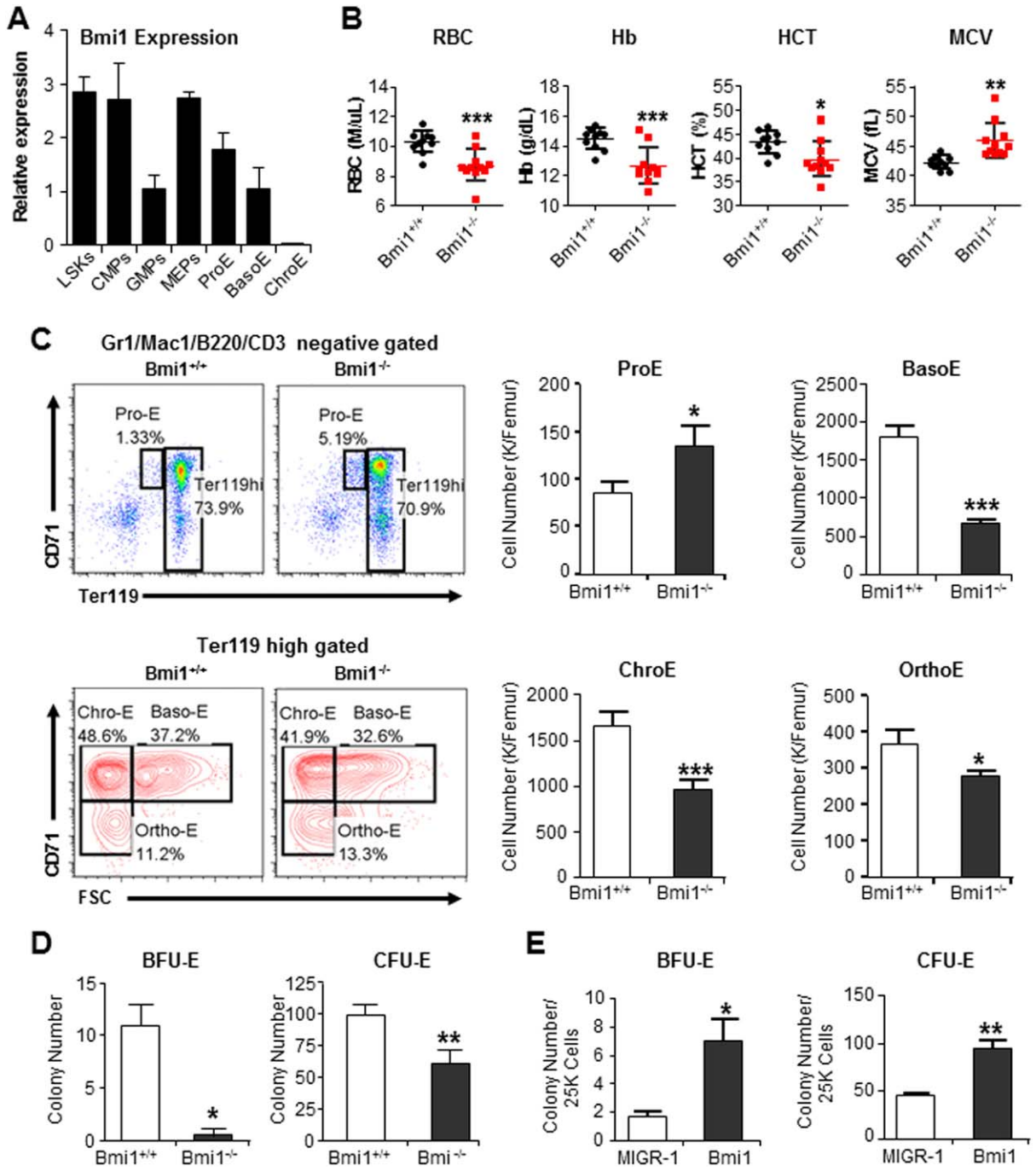


Figure 1. Bmi1 regulates mouse erythroid differentiation. **(A):** Real-time PCR analysis of *Bmi1* mRNAs in LSKs, CMPs, GMPs, MEPs, Pro-E, Baso-E, and Chro-E cells. *Bmi1* mRNA level in GMPs was arbitrarily set to 1. Data are means ± SD. Value is shown for three biological replicates. **(B):** Peripheral blood of 10–12 weeks old WT and *Bmi1* null mice were collected and analyzed. Data are means ± SD, $n = 10$ for WT mice, $n = 11$ for *Bmi1* null mice. *, $p < .05$; **, $p < .01$; ***, $p < .001$. **(C):** Erythroid progenitor frequency in the bone marrow of WT and *Bmi1* null mice was measured by CD71 and Ter119 staining in combination with cell size (FSC). Representative fluorescence-activated cell sorting profile (left panel) and the absolute number of Pro-E, Baso-E, Chro-E, and Ortho-E (right panel) in the bone marrow of wild-type and Bmi1 null mice are shown. Data are means ± SD, $n = 6$, *, $p < .05$; ***, $p < .001$. **(D):** Equal number of bone marrow mononuclear cells from WT and *Bmi1* null mice were plated on methylcellulose medium. Colonies were counted on days 2–3 (CFU-E) or days 9–10 (BFU-E). Data are means ± SD, $n = 3$, *, $p < .05$; **, $p < .01$. **(E):** Mononuclear cells from the Bmi1 null mice were infected with Bmi1 overexpressing or control retroviruses. Sorted GFP^+ cells were plated on methylcellulose medium and colonies were counted on days 2–3 (CFU-E) or days 9–10 (BFU-E). Data are means ± SD, $n = 3$, *, $p < .05$; **, $p < .01$. Abbreviations: Baso-E, basophilic erythroblasts; BFU, burst-forming unit; CFU, colony-forming unit; Chro-E, chromatophilic erythroblasts; CMPs, common myeloid progenitors; GMPs, granulocyte-macrophage progenitors; HCT, hematocrit; LSKs, Lin−Sca1+Kit+ cells; MCV, mean corpuscular volume; MEPs, megakaryocyte-erythrocyte progenitors; Pro-E, proerythroblasts.

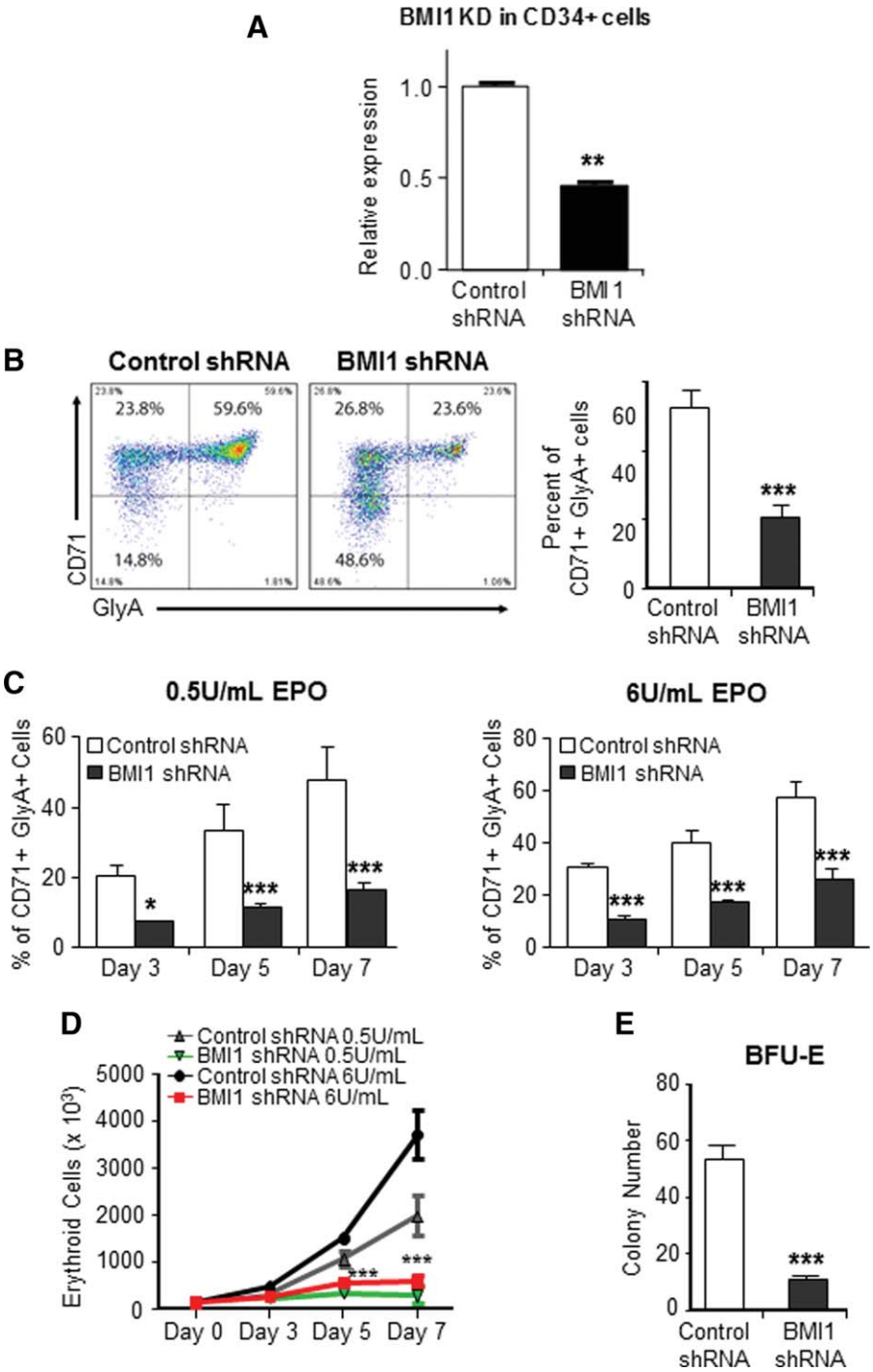
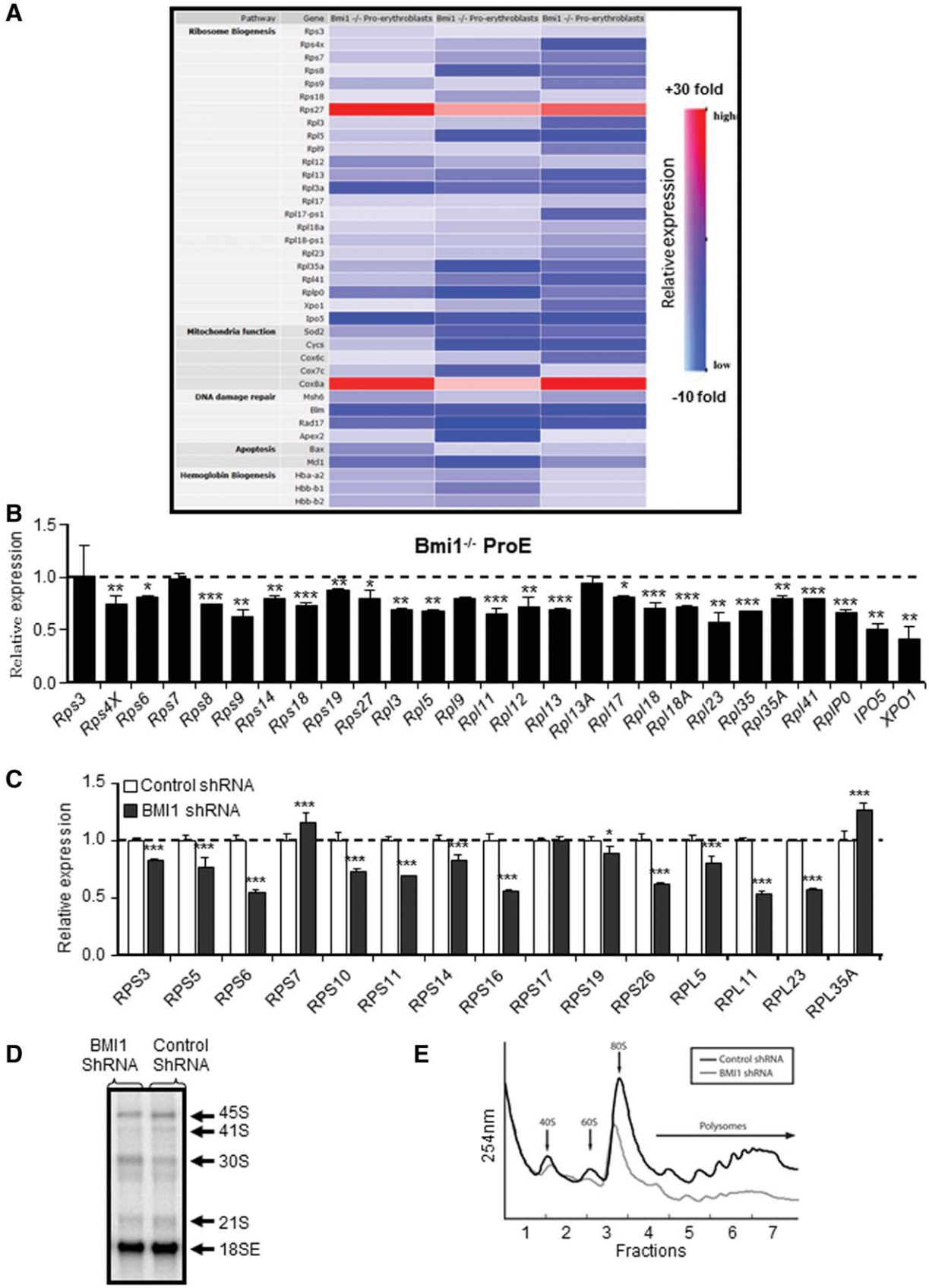


Figure 2. BMI1 regulates human erythropoiesis. **(A):** Lentiviral constructs expressing shRNAs targeting luciferase (control) or *BMI1* led to efficient knockdown of *BMI1* expression in human CB CD34$^+$ cells, as assessed by quantitative RT-PCR. Data are means ± SD. Value is shown for three biological replicates. **, $p < .01$. **(B):** Expression of CD71 and GlyA on human CD34$^+$ cells, as assayed by flow cytometric analysis at day 7 of EPO-induced culture. Data are means ± SD. ***, $p < .001$, $n = 3$. **(C):** Expression of CD71 and GlyA on human CD34$^+$ cells, cultured with different concentrations of EPO, as assayed by flow cytometric analysis at day 3, day 5, and day 7. Data are means ± SD. *, $p < .05$; ***, $p < .001$, $n = 3$. **(D):** Proliferation of control shRNA or BMI1 shRNA transduced CD34$^+$ cells at different time points after culturing in EPO-induced liquid culture. Data are means ± SD. ***, $p < .001$, $n = 3$. **(E):** 48 hours after lentiviral infection, 500 sorted GFP$^+$ CD34$^+$ cells were placed in colony-forming unit assays and the number of BFU-E colonies quantified. Data are means ± SD. ***, $p < .001$, $n = 3$. Abbreviations: BFU, burst-forming unit; EPO, erythropoietin.

software to group potential Bmi1 target genes into specific pathways that maybe important for erythropoiesis. We identified several pathways that appear to be altered in the absence of Bmi1, including ribosome biogenesis, DNA damage repair, mitochondria function, apoptosis, and hemoglobin biogenesis (Fig. 3A). Ingenuity pathway analysis indicated that ribosome biogenesis is the most altered pathway in Bmi1 null pro-E cells, manifested by downregulation of transcription of multiple


A
Pathway
Gene
Bmi1 -/- Pro-erythroblasts
Ribosome Biogenesis
Mitochondria function
DNA damage repair
Apoptosis
Hemoglobin Biogenesis
+30 fold
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low
-10 fold
B
Bmi1-/- ProE
Relative expression
Rps3
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RPS26
RPL5
RPL11
RPL23
RPL35A
D
BMI1 ShRNA
Control ShRNA
45S
41S
30S
21S
18SE
E
80S
40S
60S
Control shRNA
BMI1 shRNA
Polysomes
254nm
Fractions

Figure 3.

ribosomal protein genes (Fig. 3A). We also observed alterations in cell cycle related genes, including Rb, Cdk12, and Cdkn2a (data not shown). To verify the microarray data, we performed quantitative RT-PCR assays using both wild-type and *Bmi1* null proerythroblasts. As expected, we observed decreased expression of multiple ribosomal protein genes encoding both the small and large subunits of ribosome, including *Rps14*, *Rps19*, *Rpl11*, and *Rpl35* (Fig. 3B). Surprisingly, we did not observed decreased ribosomal protein gene expression in hematopoietic stem cells (data not shown) and immature erythroid progenitor cells ($CD71^{low}Ter119^{low}$) derived from *Bmi1* null mice compared with wild-type cells (Supporting Information Fig. S4B), indicating that Bmi1 specifically regulates ribosomal protein gene expression at the proerythroblast stage. Given that BMI1 plays an important role in human erythropoiesis, we predicted that BMI1 regulates ribosomal protein gene expression in human erythroid precursors. This was indeed the case. We found that *BMI1* deficiency results in reduced expression of multiple ribosomal protein genes in human $CD34^+$ cells (Fig. 3C). Thus, BMI1 may regulate ribosomal protein gene expression in both mouse and human erythroid progenitor cells.

Haploinsufficiency of *RPS14* and *RPS19* has been shown to disrupt pre-rRNA processing [20, 31]. To determine the effect of BMI1 deficiency on pre-rRNA processing, we performed Northern blot analysis of rRNA transcripts and found that knockdown of BMI1 in the human erythroleukemia cell line K562 had no effect on pre-rRNA processing (Fig. 3D and Supporting Information Fig. S5A, S5B) [32]. Reduced ribosomal protein gene expression has also been shown to affect the maturation of ribosome subunit and global translation efficiency [31, 33, 34]. To determine whether BMI1 regulates the maturation of ribosome subunits in erythroid progenitor cells, we performed polysome analysis using sucrose gradient centrifugation [25, 26] and observed that knockdown of *BMI1* in K562 cells significantly reduced polysome levels (Fig. 3E). Interestingly, the polysome/monosome ratios for the control shRNA and the BMI1 shRNA were roughly the same even though the polysome peaks differed drastically on the polysome profile. This indicates that there is an overall decrease in ribosomes and global translational efficiency, demonstrating that BMI1 plays a critical role in ribosome biogenesis.

Bmi1 Associates with the Promoter of Ribosomal Protein Genes and Regulates Their Expression

There are approximately 80 ribosomal proteins in humans [18, 35]. To access the association of Bmi1 with ribosomal protein genes at the genomic level, chromatin immunoprecipitation (ChIP) followed by massive massively parallel sequencing (ChIP-seq) was performed in murine L8057 megakaryoblastic cells [36]. Both Bmi1 and Ring 1b are key components of the PRC1 complex [3]. As high-quality Bmi1 antibodies are not available for ChIP-seq, we used anti-Ring1b antibody in the study. Two biological repeats were performed for each, and data were compared to control IgG [36]. We observed that Ring1b associated with the promoter of multiple ribosomal protein genes, including *Rpl7*, *Rpl10*, *Rpl22*, *Rpl29*, *Rps5*, *Rps12*, *Rps14*, and *Rps29* (Fig. 4A). It appears that Ring1b associated with the transcription start site of multiple ribosomal protein genes. To investigate whether BMI1 associates with ribosomal protein genes in human erythroid progenitor cells, we performed chromatin immunoprecipitation (ChIP) experiments using both BMI1 and RING1b antibodies. We observed that both BMI1 and RING1b binded to the promoter region of multiple ribosomal protein genes, including *RPL5*, *RPL11*, *RPL23*, *RPS14*, and *RPS19*, in K562 cells (Fig. 4B and Supporting Information Fig. S6). Moreover, we observed both BMI1 and RING1b binding sites on ribosomal protein gene promoters were enriched with active histone marks, including H3K4me3 and H3K9ac, but not the repressive H3k27me3 mark (Fig. 4C and Supporting Information Fig. S7), indicating that BMI1 may activate ribosomal protein gene expression in human erythroid cells. The core enzymatic activity of PRC1 is an E3 ubiquitin ligase activity contributed by Ring1b (and enhanced by Bmi1) which ubiquitinates histone H2A at lysine 119 [3]. We found that H2AK119ub1 mark was enriched at the promoter region of *RPS19* and knockdown of BMI1 decreased the level of H2AK119ub1 (Supporting Information Fig. S8). Furthermore, we found that knockdown of BMI1 expression decreased *RPL11* promoter activation in K562 cells (Fig. 4D). These data demonstrate that BMI1 associates with the promoter of ribosomal protein genes and positively regulates their transcription in erythroid cells.

Bmi1 Deficiency Activates the p53 Pathway in Erythroid Progenitor Cells

As ribosomal dysfunction has been shown to activate the p53 pathway [37–40], we examined whether reduced dosage of ribosomal protein genes in *Bmi1* null erythroid progenitor cells activates p53. While we observed no change in p53 mRNA levels, p53 target gene *p21* was upregulated in *Bmi1* null Pro-E cells compared with wild-type cells (Fig. 5A). We also observed that knockdown of BMI1 in human $CD34^+$ cells increased p21 expression without altering p53 mRNA levels as shown in Figure 5B, indicating that p53 protein may accumulate in the absence of Bmi1. As expected, we observed that both p53 and p21 proteins were accumulated in Bmi1 null BM cells compared with

Figure 3. Bmi1 deficiency results in decreased expression of some ribosomal protein genes in erythroid progenitor cells. **(A):** Transcript profiling of Pro-E cells from WT and $Bmi1^{-/-}$ mice was analyzed by Agilent Whole Mouse Genome Oligo Microarrays. Genes whose expressions are increased or decreased more than twofold in $Bmi1^{-/-}$ Pro-E cells compared to wild-type cells are shown. Data shown are relative expression as compared to WT Pro-E cells (set as 1). We used ingenuity pathways analysis (Ingenuity Systems) to group genes into specific canonical pathways. Values are shown for three biological replicates. **(B):** Real-time RT-PCR analysis of ribosomal protein gene expression in Pro-E cells from WT and $Bmi1^{-/-}$ mice. Data shown are relative expression as compared to WT Pro-E cells (set as 1). Data are means $\pm$ SD. Value is shown for three biological replicates. *, $p < .05$; **, $p < .01$;***, $p < .001$. **(C):** Real-time RT-PCR analysis of expressions of different ribosomal protein genes in transduced human $CD34^+$ cells. Data shown are relative expression as compared to control shRNA transduced cells (set as 1). Data are means $\pm$ SD. Value is shown for three biological replicates. *, $p < .05$; ***, $p < .001$. **(D):** Northern blot analysis of total RNA from K562 cells infected with control or *BMI1* shRNA. **(E):** Knockdown of BMI1 in K562 cells reduces polysome levels. Representative polysome profiles of K562 cells infected with control or *BMI1* shRNAs by sucrose gradient centrifugation are shown.

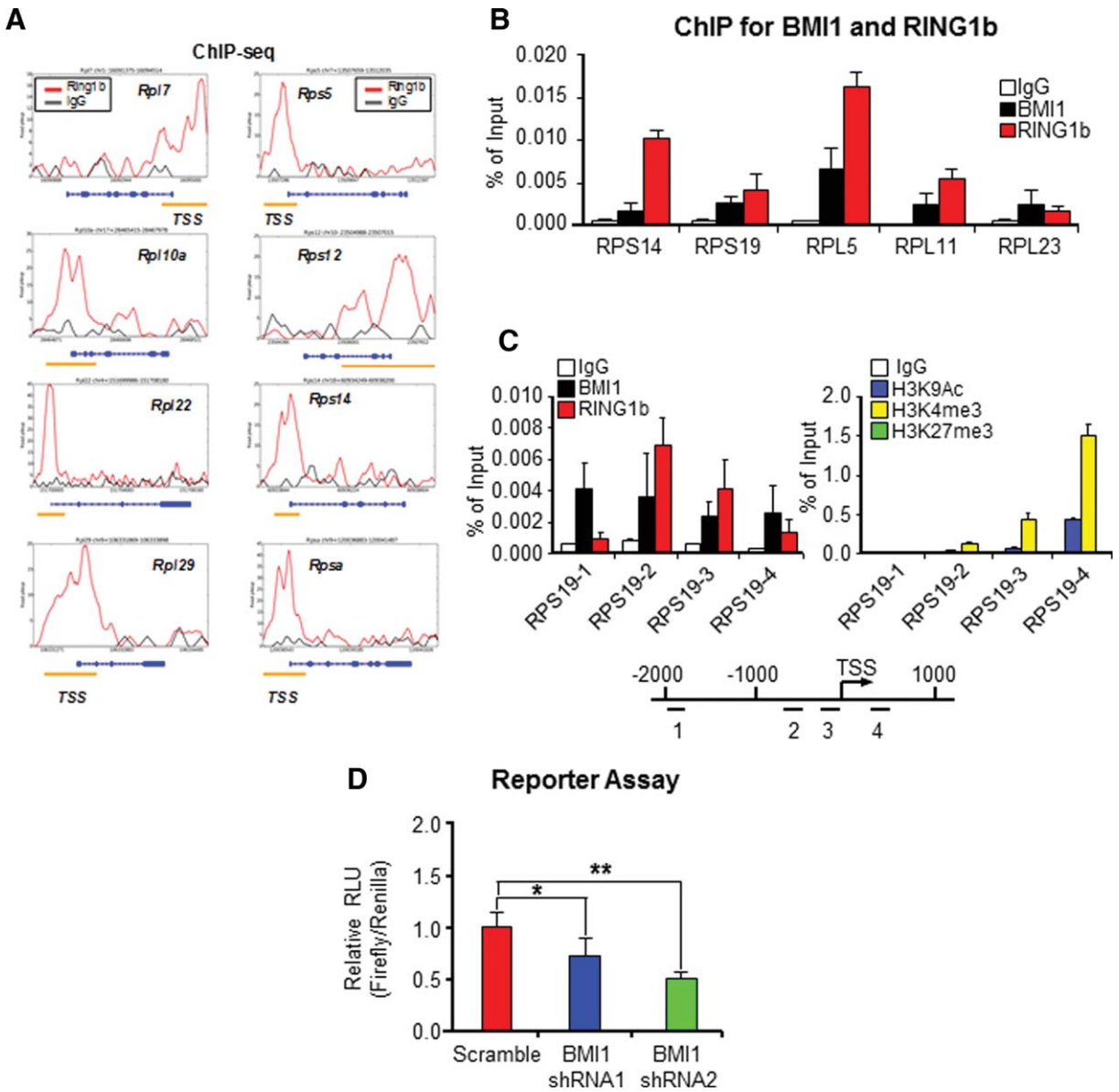

Figure 4. Bmi1 associates with the promoter of some ribosomal protein genes and enhances their expression. **(A):** Representative Ring1b and control IgG ChIP-seq profiles of loci occupied by Ring1b in murine L8057 megakaryoblastic cells. It appears that Ring1b associates with the TSS of multiple ribosomal protein genes. **(B):** BMI1 and RING1b associates with the promoter of some ribosomal protein genes in vivo. Chromatin-bound DNA from K562 cells was immunoprecipitated with a BMI1-antibody, a RING1b-antibody, or with normal mouse IgG. qPCR amplification was performed on corresponding templates using primers for *RPS14*, *RPS19*, *RPL5*, *RPL11*, and *RPL23* genes. **(C):** Chromatin-bound DNA from K562 cells was immunoprecipitated with a BMI1-antibody, a RING1B-antibody, a H3K9ac-antibody, a H3K4me3-antibody, a H3K27me3-antibody, or with normal mouse IgG. qPCR amplification was performed on corresponding templates using primers for *RPS19* gene. **(D):** Knockdown of BMI1 decreases the activation of the *RPL11* promoter. K562 cells with reduced *BMI1* expression were transfected with *RPL11* promoter-driven luciferase plasmid. Luciferase activity was assayed 24 hours after transfection. Values are means ± SD, $n = 3$, *, $p < .05$; **, $p < .01$. Abbreviations: ChIP, chromatin immunoprecipitation; TSS, transcription start site.

wild-type cells (Fig. 5C), demonstrating loss of Bmi1 stabilizes p53 protein. We also found that Bmi1 target gene $p19^{Arf}$ expression was upregulated in both Bmi1 null pro-E cells and BMI1 KD $CD34^+$ cells (Supporting Information Fig. S9A, S9B).

Elevated p53 protein levels could induce apoptosis and/or cell cycle arrest. To assess the effect of Bmi1 deficiency on the survival of erythroid cells, we assayed for apoptosis of erythroid progenitor cells by Annexin-V staining. While immature erythroid progenitor cells ($CD71^-Ter119^-$ cells) from the Bmi1 null mice exhibited normal apoptosis, there was a modest increase of apoptotic pro-E cells, although not statistically significant (Fig. 5D). Next, we examined the cell cycle status of $CD71^-Ter119^-$ and Pro-E cells by propidium iodide (PI) staining. Loss of Bmi1 did not affect cell cycle status of $CD71^-Ter119^-$ cells (Supporting Information Fig. S10); however, we observed that Bmi1 null pro-E cells were arrested at the G0/G1 phase of the cell cycle (Fig. 5E). Consistent with data shown in Figure 5E, knockdown of *BMI1* significantly increased the proportion of $CD34^+$ cells in the G0/G1 phase of the cell cycle, compared to that of the control shRNA infected cells (Fig. 5F). Thus, Bmi1 deficiency activates the p53 pathway, resulting in cell cycle arrest.

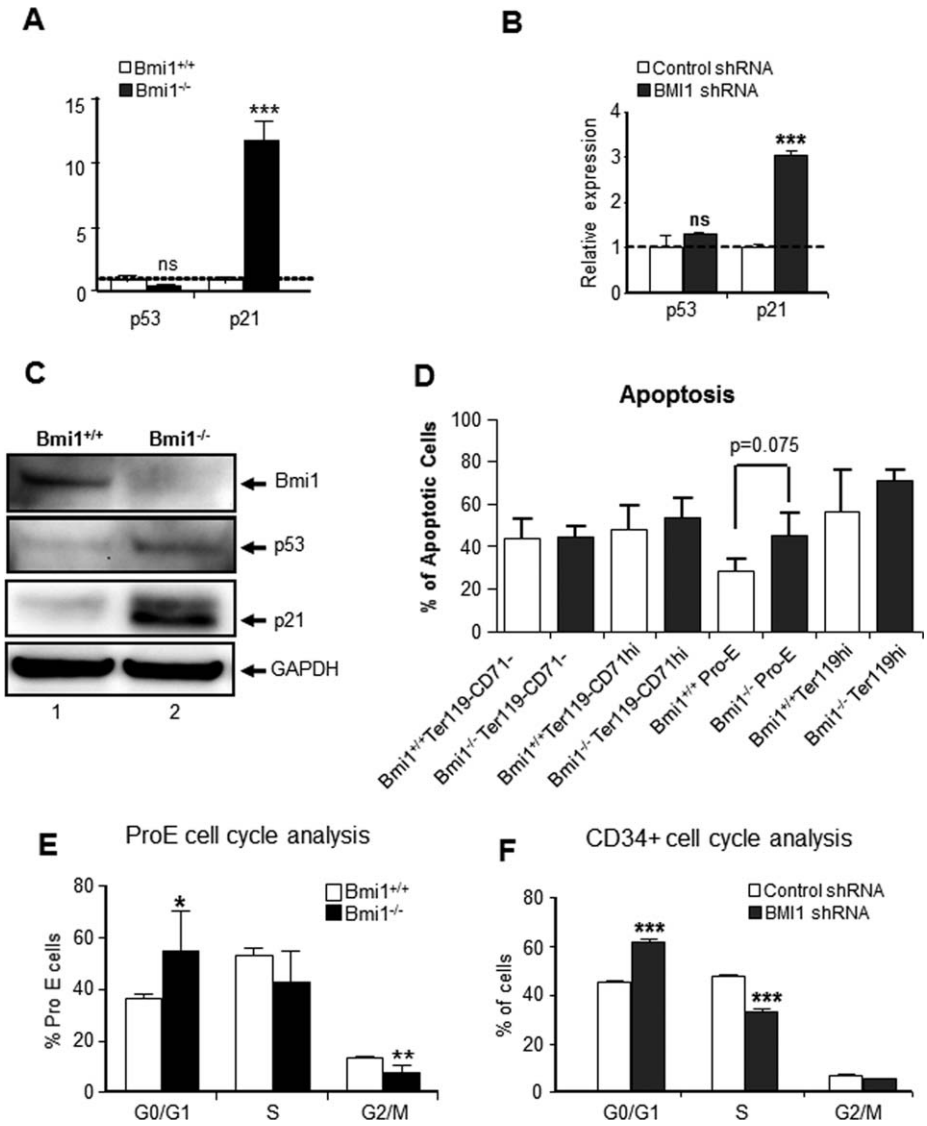

Figure 5. Loss of Bmi1 leads to activation of the p53 pathway in erythroid progenitor cells. **(A):** Real-time RT-PCR analysis of *p53* and *p21* expression in proerythroblasts ($CD71^{high}Ter119^{low}$) from the bone marrow of WT and Bmi1 null mice. Data shown are relative expression as compared to WT Pro-E cells (set as 1). Data are means ± SD. Value is shown for three biological replicates. ***, $p < .001$. **(B):** Knockdown of *BMI1* expression in human CB $CD34^{+}$ cells upregulates p21 expression as assessed by quantitative RT-PCR. Data are means ± SD. Value is shown for three biological replicates. ***, $p < .001$. **(C):** p53 and p21 proteins in bone marrow mononuclear cells from both wild-type and Bmi1 null mice were assessed by Western blot analysis. **(D):** Annexin V staining of bone marrow cells isolated from WT and Bmi1 null mice. Cells were cultured without cytokine for 24 hours. Data are means ± SD ($n = 3$). **(E):** Cell cycle analysis of erythroblasts from the bone marrow of WT and Bmi1 null mice. Data are means ± SD, *, $p < .05$, **, $p < .01$, $n = 3$. **(F):** Cell cycle analysis of control shRNA or BMI1 shRNA transduced $CD34^{+}$ cells after 7 days in EPO-induced liquid culture. Data are means ± SD. ***, $p < .001$, $n = 3$.

Genetic Inhibition of p53 Activity Rescues the Erythroid Defects Seen in the Bmi1 Null Mice

Deletion of p53 can rescue the erythroid defects in animal models of DBA and 5q− syndrome, suggesting a p53-dependent mechanism underlies the pathophysiology of DBA and 5q− syndrome [38–40]. To examine whether inhibiting p53 function rescues the erythroid defects seen in *Bmi1* null mice, we used a dominant negative mutant form of p53, $p53^{R248W}$, which has been shown to inhibit wild-type p53 functions [41]. The $p53^{R248W}$ mice express human p53 mutant protein from the endogenous murine *Trp53* promoter [22]. We generated $Bmi1^{-/-}p53^{R248W}$ double mutant mice and analyzed the hematopoietic phenotype in a cohort of four genotypes ($Bmi1^{+/+}p53^{+/+}$; $Bmi1^{+/+}p53^{R248W}$; $Bmi1^{-/-}p53^{+/+}$; and $Bmi1^{-/-}p53^{R248W}$). We observed that the red blood cell (RBC), hemoglobin (Hb), and hematocrit (HCT) levels were rescued in $Bmi1^{-/-}p53^{R248W}$ mice (Fig. 6A). To examine whether dominant-negative p53 can also rescue defective erythropoiesis in *Bmi1* null mice, we examined the frequency of erythroid progenitor cells in these mice. While loss of Bmi1 increased the frequency of Pro-E cells, inhibiting p53 activity with mutant p53 returned the number of these cells in the BM to normal (Fig. 6B). We also performed CFU assays and found that inhibiting p53 activity in *Bmi1* null BM cells increases their ability to form both BFU-E and CFU-E colonies (Fig. 6C). We also found that inhibiting p53 activity enhanced myeloid differentiation of Bmi1 null cells (Supporting Information Fig. S11). In addition, we

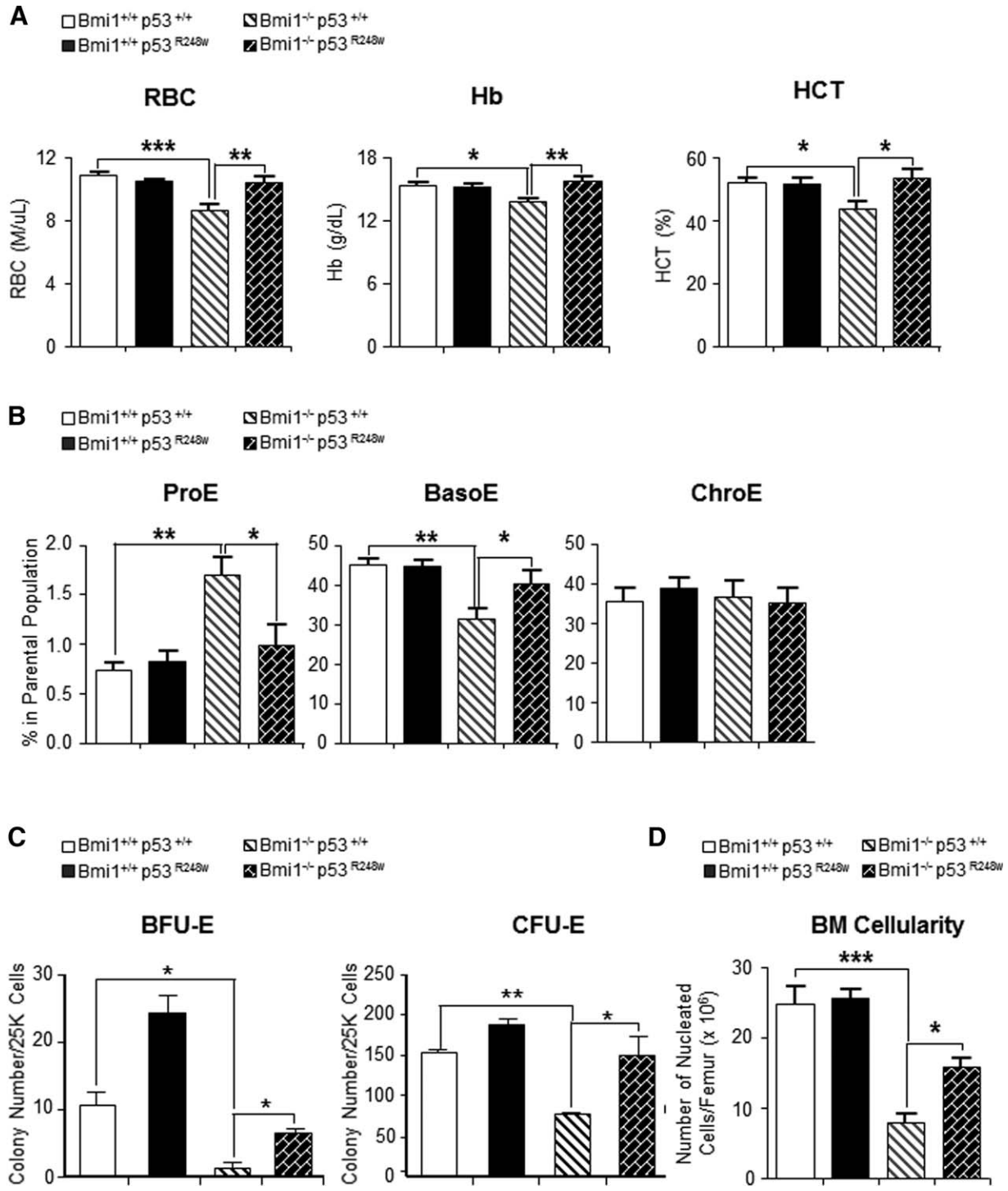

Figure 6. Genetic inhibition of p53 activity rescues the erythroid defects seen in the Bmi1 null mice. **(A):** Peripheral blood from 10 to 12 weeks old mice with indicated genotype was collected and analyzed. Data are means ± SD, *, $p < .05$; **, $p < .01$; ***, $p < .001$, $n = 3–8$ mice per group. **(B):** Erythroid progenitor frequency in the BM of mice with indicated genotype was measured by CD71 and Ter119 staining in combination with cell size (FSC). Data are means ± SD, $n = 3–8$ mice per group, *, $p < .05$; **, $p < .01$. **(C):** Equal number of bone marrow mononuclear cells from four genotype mice were plated on methylcellulose medium supplemented with EPO (CFU-E) or EPO, SCF, and IL-3 (BFU-E) in triplicates (25K cells/well). Colonies were counted on days 2–3 (CFU-E) or days 9–10 (BFU-E). Data are means ± SD, $n = 3$, *, $p < .05$; **, $p < .01$. **(D):** Total nucleated cells per femur from 10 to 12 weeks old mice were counted. Data are means ± SD, $n = 3–8$. *, $p < .05$; ***, $p < .001$. Abbreviations: BFU, burst-forming unit; BM, bone marrow; CFU, colony-forming unit; HCT, hematocrit.

found that inhibiting p53 activity returned p21 expression to normal (Supporting Information Fig. S12A). While expression of dominant-negative p53 rescued the cell cycle arrest of Bmi1 null cells (Supporting Information Fig. S12B), inhibiting p53 activity had no effect on ribosomal protein gene expression in these cells (Supporting Information Fig. S13). These data demonstrate that Bmi1 regulates ribosomal protein gene expression in a cell cycle status-independent manner.

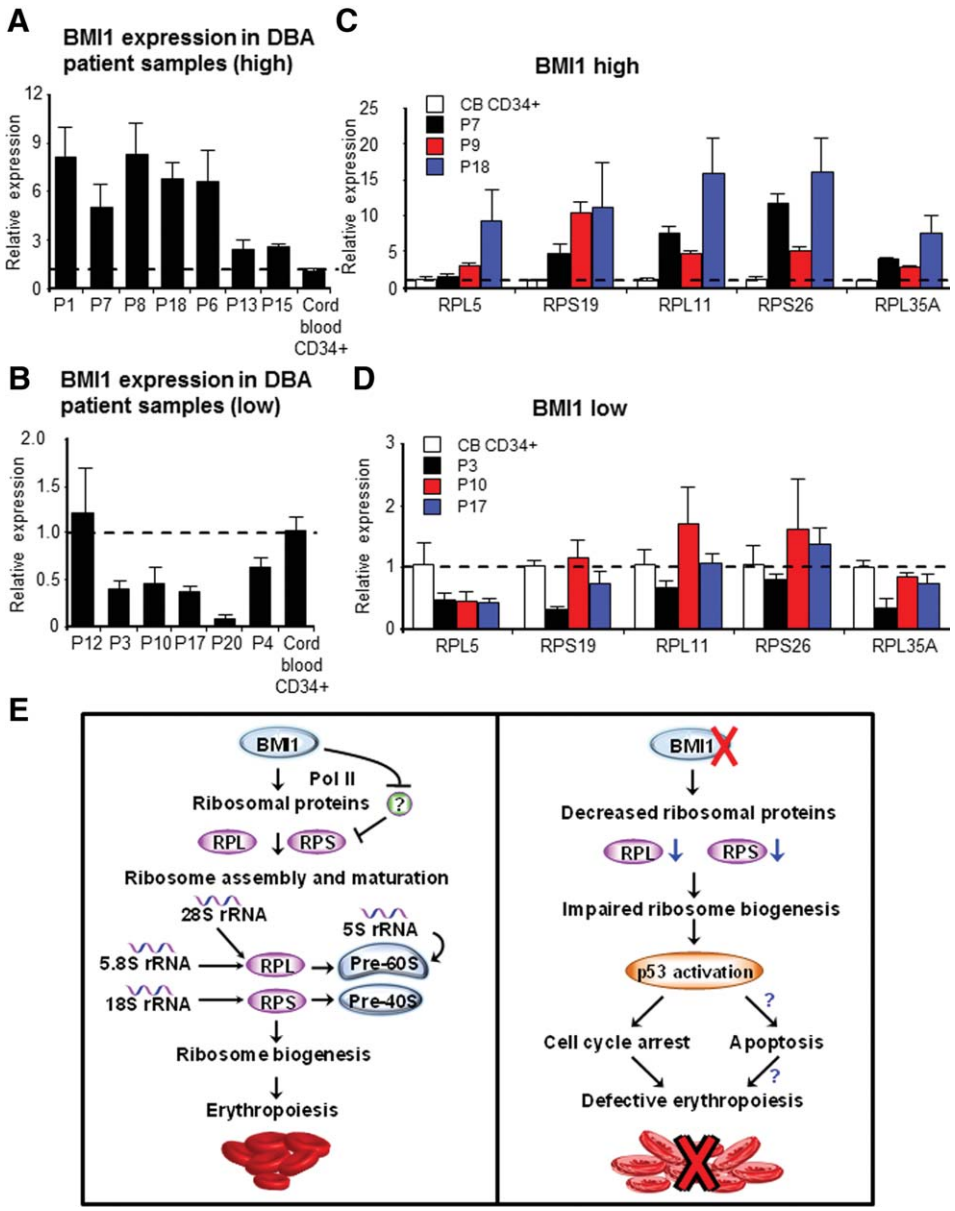

Figure 7. *BMI1* is differentially expressed in CD34$^+$ cells from patients with DBA. **(A):** Increased *BMI1* expression in CD34$^+$ cells from some DBA patient samples as assayed by quantitative RT-PCR assays. BMI1 mRNA levels in normal human cord blood CD34$^+$ cells were arbitrarily set to 1. **(B):** Reduced *BMI1* expression in CD34$^+$ cells from some DBA patient samples as assayed by quantitative RT-PCR assays. **(C):** The expression of some ribosomal protein genes in DBA patient samples with high *BMI1* expression. Ribosomal protein gene expression levels in normal human cord blood CD34$^+$ cells were arbitrarily set to 1. **(D):** Ribosomal protein gene expression in DBA patient samples with low *BMI1* expression. **(E):** A schematic model showing how BMI1 regulates ribosome biogenesis in erythroid progenitor cells. BMI1 associates with the promoter of ribosomal protein genes and positively regulate their expression. BMI1 promotes erythroid development through regulating ribosome biogenesis in erythroid progenitor cells (left panel). In the absence of BMI1, ribosome biogenesis is impaired. Defective ribosome biogenesis activates the p53 pathway, resulting in cell cycle arrest and impaired erythroid differentiation (right panel). Our work indicates that BMI1 deficiency may contribute to the pathogenesis of ribosomopathies, including DBA. Abbreviation: DBA, Diamond-Blackfan anemia.

Bmi1 null mice develop hypocellular BM and show decreased BM cellularity over time [13]. We found that the decreased BM cellularity seen in the *Bmi1* null mice was partially rescued in *Bmi1*$^{-/-}$*p53*^{R248W} mice (Fig. 6D). Although the Bmi1 null mice have reduced body size [13], inhibiting p53 does not increase the body weight of Bmi1 null mice (Supporting Information Fig. S14). In summary, we show that genetic inhibition of p53 activity rescues the erythroid defects seen in *Bmi1* null mice, indicating that activation of the p53 pathway impairs erythropoiesis in *Bmi1* null mice.

BMI1 Is Differentially Expressed in CD34$^+$ Cells Isolated from Patients with DBA

We demonstrated that *Bmi1* null mice recapitulate several critical features of human DBA, including impaired ribosome biogenesis, activation of the p53 pathway, cell cycle arrest, and defective erythropoiesis, indicating that BMI1 deficiency

may contribute to the pathogenesis of DBA. Therefore, we examined human *BMI1* gene expression in $CD34^+$ cells isolated from DBA patients using quantitative RT-PCR assays. While 7 out of 13 DBA samples had high *BMI1* expression compared with normal human cord blood $CD34^+$ cells (Fig. 7A), 5 out of 13 DBA samples had low *BMI1* expression (Fig. 7B). Moreover, we observed that high *BMI1* expression correlates with increased expression of some ribosomal protein genes, including *RPL5*, *RPL11*, *RPL35A*, *RPS19*, and *RPS26* (Fig. 7C), whereas low *BMI1* expression correlates with decreased expression of some ribosomal protein genes (Fig. 7D). These data suggest that BMI1 may play a pathological role in DBA.

Discussion

We found that Bmi1 is highly expressed in erythroid progenitor cells, where it functions as an enhancer of erythroid differentiation. In these cells, Bmi1 associates with some ribosomal protein genes and enhances their expression. As erythroid progenitor cells undergo differentiation, Bmi1 expression decreases, thereby decreasing the transcription of ribosomal protein genes and cell proliferation (Fig. 7E, left panel). The Bmi1 gene expression pattern we identified is consistent with Bmi1 expression pattern shown in the ErythronDB [42]. Loss of Bmi1 results in downregulation of transcription of multiple ribosomal protein genes, decreased protein synthesis, activation of the p53 pathway, cell cycle arrest, and defective erythroid differentiation (Fig. 7E, right panel).

To understand how Bmi1 regulates erythropoiesis, we performed gene expression profiling assays to identify genes and pathways regulated by Bmi1. Several pathways appear to be altered in the absence of Bmi1, including ribosome biogenesis, mitochondria function, DNA damage repair, apoptosis, and hemoglobin biogenesis. Based on ingenuity pathway analysis, the ribosome biogenesis pathway was the most altered pathway in Bmi1 null pro-E cells. Given that impaired ribosome biogenesis underlies the pathogenesis of DBA and 5q− syndrome [18–21], we decided to focus our studies on the ribosome biogenesis pathway. There are approximately 80 ribosomal proteins in mammalian cells [18, 35]. While the expression of approximately 20 ribosomal protein genes was significantly downregulated in Bmi1 null proerythroblasts, the expression of the majority of ribosomal protein genes was unchanged (data not shown), suggesting that there is an imbalanced synthesis of ribosomal proteins in the absence of Bmi1 [18, 19, 43]. Germline mutations in *RPS19* and other genes encoding ribosomal proteins cause DBA [18, 19]. The 5q− syndrome is caused by a somatically acquired deletion of chromosome 5q, which leads to haploinsufficiency of the ribosomal protein RPS14 and an erythroid phenotype highly similar to DBA [18–20]. Global gene expression analysis of DBA patients has shown that *RPS19* mutations lead to downregulation of transcription of multiple ribosomal protein genes [44]. Further studies of highly purified HSCs from healthy patients and those with MDS links reduced expression of several ribosomal protein genes to decreased RBC maturation [40]. Thus, it is likely that downregulation of multiple ribosomal protein genes in Bmi1 null erythroid precursors impairs ribosome biogenesis.

To investigate whether Bmi1 regulates ribosome biogenesis, we performed genome wide association studies and ChIP experiments. We demonstrated that Bmi1 associates with several ribosomal protein genes and positively regulates their expression in erythroid progenitor cells. In ChIP experiments, BMI1 appears to bind to the promoter region of some ribosomal protein genes with enriched active histone marks, including H3K4me3 and H3K9Ac, but not the repressive H3K27me3 mark. We found that H2AK119ub1 mark was enriched at the promoter region of *RPS19* and knockdown of BMI1 decreased the level of H2AK119ub1, indicating that the PRC1 complex may regulate ribosomal protein gene expression in erythroid progenitor cells. BMI1 is a known epigenetic repressor in hematopoietic stem and progenitor cells [3, 13]; however, our data indicate that it may function as an activator of ribosomal protein gene expression in erythroid progenitor cells. Polycomb proteins are found in most cells, but they must be targeted to specific genes in specific cell types to regulate pluripotency and differentiation [1–3]. MYC is a direct regulator of ribosome biogenesis in multiple cell types [45]. Given that BMI1 cooperates with MYC to promote the generation of B- and T-cell lymphomas [46, 47], we hypothesize that MYC recruits BMI1 onto the promoter of ribosomal protein genes and assembles an activating complex, enhancing ribosomal protein gene transcription. It is also possible that BMI1 inhibits a negative regulator of ribosome biogenesis and indirectly regulates this process.

Given that ribosomal dysfunction can activate the p53 pathway [39, 40], we reasoned that reduced ribosomal protein gene expression in Bmi1 null erythroid progenitor cells may activate the p53 pathway. This was indeed the case. While p53 mRNA levels were comparable, p53 proteins were elevated in Bmi1 null BM cells, indicating that Bmi1 deficiency stabilizes p53 protein. In addition, we observed that p53 target gene p21 was upregulated in Bmi1 null erythroid progenitor cells, demonstrating that the p53 pathway is activated in the absence of Bmi1. In response to ribosomal stress, several ribosomal proteins, including RPL5 and RPL11, bind to MDM2 and block MDM2-mediated p53 ubiquitination and degradation, resulting in p53-dependent cell cycle arrest [39–41, 48]. It is possible that similar mechanisms may apply to the activation of p53 pathway in Bmi1-deficient cells. Bmi1 is a potent negative regulator of the Ink4a-Arf locus, which encodes the cell cycle regulator and tumor suppressor $p16^{Ink4a}$ and $p19^{Arf}$ proteins [3]. p19 interacts with Mdm2 and stabilizes the p53 protein [1–3]. We found that p19 expression is upregulated in Bmi1-deficient cells, suggesting accumulation of p53 protein may also be due to increased expression of $p19^{Arf}$.

Deletion of p53 in mouse models of human DBA and 5q− syndrome can rescue the erythroid defects [34, 38–40]. We found that inhibiting p53 activity with a dominant-negative mutant p53 partially rescues the erythroid defects of the *Bmi1* null mice, suggesting that a p53-dependent mechanism underlies the pathophysiology of anemia. Human K562 erythroleukemia cells have been used to study the impact of *RPS19* mutations on ribosome biogenesis [32]. Therefore, we used these cells investigate how BMI1 regulates ribosome biogenesis. We found that knockdown of *BMI1* expression in K562 cells results in downregulation of multiple ribosomal protein genes as we observed in human $CD34^+$ cells (data not shown). Given that K562 cells are p53 null, our data suggest that BMI1 regulates ribosomal protein gene expression in a p53-independent manner.

Stabilization of p53 leads to cell cycle arrest and/or apoptosis in mouse model of human DBA and 5q− syndrome [34, 38, 40]. In addition, haploinsufficiency for ribosomal protein gene activates the p53 pathway in human erythroid progenitor cells, leading to accumulation of p21 and consequent cell cycle arrest [39]. We observed that Bmi1 deficiency in both mouse and human erythroid progenitor cells results in upregulation of p21 and G0/G1 cell cycle arrest. Bmi1 null proerythroblasts appear to have higher rate of apoptosis, but the difference is not statistically significant. In addition, we found that antiapoptotic gene *Mcl1* was downregulated in Bmi1 null cells (Fig. 3A), indicating that enhanced apoptosis may play a role in the erythroid defect seen in Bmi1 null mice. Given that cell cycle progression and ribosomal biogenesis is coupled [48], the reduced ribosomal protein gene expression in Bmi1 null erythroid precursors could also be due to cell cycle arrest. While inhibiting p53 activity using dominant-negative p53 rescued the cell cycle arrest of Bmi1 null cells, inhibiting p53 activity had no effect on ribosomal protein gene expression in these cells, suggesting that Bmi1 regulates ribosome biogenesis in a cell-cycle independent manner.

Although peripheral blood counts from 7 to 8 weeks old Bmi1 null mice were close to normal (data not shown), these mice started to show anemic features from 10 to 12 weeks old, manifested by reduced RBC, HB, and HCT levels compared with wild-type mice. Interestingly, we observed that MCV levels are increased in Bmi1-deficient mice, which is a key feature of DBA and 5q− syndrome [18–21]. Rps19-deficient mice develop a macrocytic anemia together with leukocytopenia, leading to the exhaustion of hematopoietic stem cells and BM failure [34]. In a mouse model of human 5q− syndrome, haploinsufficiency of the Cd47-Nid67 interval (containing *Rps14*) caused macrocytic anemia and prominent erythroid dysplasia in the BM [38]. Thus, Bmi1 deficiency may link to the pathogenesis of ribosomopathies, including DBA and 5q− syndrome.

Recent advances in characterizing the genetic abnormalities underlying DBA have demonstrated mutations or deletions of genes encoding both large (RPL) and small (RPS) ribosomal subunit proteins in 50%–60% of affected patients [49, 50]. We reported clinical analysis of 45 cases of DBA in China and found that clinical features of these Chinese DBA patients are similar to other ethical groups [51]. We performed mutational analysis of ribosomal protein genes in 21 cases of DBA as well [52]. However, genetic abnormalities in the remaining DBA patients are largely unknown. We observed decreased expression of multiple ribosomal protein genes encoding both the small and large subunits of ribosome in Bmi1 null erythroid progenitor cells, including *Rps14*, *Rps19*, *Rps27*, and *Rpl11*, which has been implicated in the pathogenesis of DBA and 5q− syndrome [18–20]. Moreover, the erythroid defects seen in *Bmi1* null mice recapitulate several, but not all clinical features of human DBA and 5q− syndrome [18–20]. In this study, we found that *BMI1* expression in $CD34^+$ cells from patients with DBA correlates with the expression of some ribosomal protein genes in a small cohort of DBA patients. While there is no direct link between BMI1 and the pathogenesis of DBA, our data suggest that BMI1 may play a pathological role in DBA and other ribosomopathies. Exome sequencing has identified GATA1 mutations in DBA patients without ribosomal protein gene mutations [53]. Reduced *BMI1* expression in patients with DBA may be due to mutations. In the future, we will perform exome sequencing to detect *BMI1* mutations in DBA patients without ribosomal protein gene mutations.

Conclusion

In summary, this study reveals a critical role of Bmi1 in erythroid development and ribosome biogenesis. Our results suggest that BMI1 may play a pathological role in DBA and other ribosomopathies.

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Author Contributions

R.G., S.C., and Y.L.: contributed to design, experimental performance, and interpretation and writing; M.K., H.Y., Y.Z., Y.W., S.Y., A.S., M.Y., and S.V.: contribute to experimental performance; E.F., A.C., Y.X., M.C., Y.A., R.K., R.W., S.R., and X.Z.: contributed to design, interpretation, and writing.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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Targeting Mutant p53 to Improve Leukemia Treatment

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Acute myeloid leukemia (AML) is the most common acute leukemia in adults. When AML presents in patients at age 60 or above, or at any age after toxic chemical exposure, the frequency of *TP53* gene mutation is almost 20% and carries a very poor prognosis, with most patients living less than 18 months. Unfortunately, little progress has been made in treating these AML patients over the past 4 decades. There remains a critical need to identify therapeutic agents that would be effective in treating AML patients with mutant p53 and improve leukemia treatment. We have been investigating the role of tumor suppressor p53 in regulating hematopoietic stem cell (HSC) behavior and defined a critical role of p53 in regulating HSC self-renewal and response to genotoxic stresses. While the tumor suppressor p53 is a critical regulator of HSC behavior, the role of mutant p53 in the pathogenesis of AML remains elusive. To define the role of mutant p53 in hematopoiesis, we introduced 9 hot-spot p53 mutants identified in AML, including $p53^{R248W}$, $p53^{R273H}$ and $p53^{Y220C}$, into wild type hematopoietic cells using retrovirus-mediated transduction and investigated the role of these p53 mutants in regulating HSC self-renewal. We found that hematopoietic cells expressing $p53^{R248W}$, $p53^{Y220C}$ and $p53^{R273H}$ show enhanced repopulating potential 16 weeks following transplantation, indicating that these mutant p53 proteins enhance HSC self-renewal *in vivo*. As the codon 248 of p53 protein is most frequently mutated in AML, we decided to investigate the role of $p53^{R248W}$ mutant in leukemogenesis by using the humanized knock-in mice of $p53^{R248W}$. We performed competitive repopulation assays and found that the repopulating ability of $p53^{R248W}$ cells was 2 folds higher than that of the wild type cells at 16 weeks post transplantation, demonstrating that the $p53^{R248W}$ mutant enhances HSC self-renewal in vivo. A large fraction of human tumors carry *TP53* mutations that abolish transcriptional activation of p53 target genes. Reconstitution of wild-type p53 in vivo triggers rapid elimination of tumors. Therefore, pharmacological reactivation of mutant p53 is a promising strategy for novel cancer therapy. Small molecule PRIMA-1 has been shown to restore wild-type conformation to some mutant p53 proteins and induce apoptosis in human tumor cells. We found that hematopoietic cells expressing mutant p53 are sensitive to PRIMA-1 treatment and undergo p53-dependent apoptosis. Furthermore, we found that PRIMA-1 inhibits the growth of primary human AML cells with *TP53* mutation in a dosage-dependent manner. Thus, we demonstrate that mutant p53 enhances hematopoietic stem cell self-renewal and pharmacological inhibition of mutant p53 sensitizes the drug-resistant leukemia stem cells (LSCs) to chemotherapy and improves leukemia treatment.

Bmi1 maintains the self-replenishing ability of B-1a cells in postnatal mice.

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Mouse B-1 cells are innate immune cells that secrete natural antibodies and play important roles in the fist line of defense to bacterial infection. B-1 cells display an $^{+}IgM^{hi}IgD^{lo}CD11b^{+}$ phenotype, and preferentially localize within the peritoneal cavity. $CD5^{+}$B-1a cells are considered to be of fetal origin since adult BM HSCs fail to reconstitute B-1a cells while fetal liver cells reconstitute B-1a cells in irradiated recipient mice. We have recently demonstrated that YS cells at E9.5 (at the time prior to HSC emergence in the mouse embryo) are the earliest origin of B-1a cells. The mechanisms that permit B-1a cells to undergo self-replenishment in vivo in the absence of ongoing stem cell input remain unclear.

Bmi1 is a polycomb group protein that is known to be critical for self-renewal of adult BM HSCs and neural stem cells. In *Bmi1*$^{-/-}$ postnatal BM, the number of HSCs was markedly reduced and long-term reconstituting ability was lost, while enforced expression of *Bmi1* in HSCs promoted their self-renewal ability. We hypothesized that Bmi1 is critical for B-1a cell self-replenishment and examined B-1 cell populations in Bmi1$^{-/-}$ embryonic and adult mice.

Bmi1 mRNA expression was higher in B-1a cells than other lymphoid subsets in wild type mice. In *Bmi1*$^{-/-}$ mice, the B-1a cell population was significantly ($p<0.05$) decreased in the percentage (4 fold less than WT) and total cell number (50 fold less than WT). On the contrary, the percentage and number of B-1 specific progenitors ($lin^{-}AA4.1^{+}CD19^{+}$B220dim cells) was increased in the E14-15 fetal liver of Bmi1$^{-/-}$ embryos. When the peritoneal cells from adult Bmi1$^{-/-}$ mice were transferred into the peritoneal cavity of sublethally irradiated NOG neonates, cells only transiently engrafted, and no long-term reconstitution was observed. Wild type peritoneal cells reconstituted B-1a cells for 4-9 months as previously reported. When Bmi1$^{-/-}$ fetal liver cells were rescued for Bmi1 expression by retroviral gene transfer and transplanted, B-1a cells were recovered in the recipient mice **>** 4 months after transplantation at numbers equivalent to wild type transplanted cells. Thus, Bmi1 plays an important role in the self-replenishing ability of B-1a cells in the postnatal mouse.

PRL2 maintains hematopoietic stem and progenitor cells through regulating SCF/KIT signaling

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The phosphatase of regenerating liver family of phosphatases, consisting of PRL1, PRL2 and PRL3, represents an intriguing group of proteins implicated in cell proliferation and tumorigenesis. However, the role of PRLs in normal and malignant hematopoiesis is largely unknown. While SCF/KIT signaling plays an important role in hematopoietic stem and progenitor cell (HSPC) maintenance, how SCF/KIT signaling is regulated in HSPCs remains poorly understood. We here report that PRL2 regulates HSPC maintenance through regulating SCF/KIT signaling. To define the role of PRL2 in hematopoiesis, we analyzed the hematopoietic stem cell (HSC) behavior in *Prl2* deficient mice generated by our group. *Prl2* deficiency results in ineffective hematopoiesis and impairs the long-term repopulating ability of HSCs. In addition, *Prl2* null HSPCs are less proliferative and show decreased colony formation in response to SCF stimulation. Moreover, *Prl2* null HSPCs show reduced activation of the PI3K/AKT and ERK signaling in steady state and following SCF stimulation. Importantly, we found that the ability of PRL2 to enhance SCF signaling depends on its enzymatic activity, demonstrating that PRL2 mediates SCF/KIT signaling in HSPCs. Thus, PRL2 plays a critical role in hematopoietic stem and progenitor cell maintenance through regulating SCF/KIT signaling. Furthermore, loss of *Prl2* decreased the ability of oncogenic KITD814V mutant in promoting hematopoietic progenitor cell proliferation and in activation of signaling pathways. We also checked the expression of PRL2 proteins in human AML cell lines and found increased level of PRL2 proteins in some acute myeloid leukemia (AML) cells compared with normal human bone marrow cells, indicating that PRL2 may play a pathological role in AML. Our results suggest that the PRL2 phosphatase may be a druggable target in myeloproliferative disease (MPD) and acute myeloid leukemia (AML) with oncogenic KIT mutations.

BMI1 promotes erythropoiesis through regulating ribosome biogenesis

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Diamond-Blackfan anemia (DBA) is a rare congenital bone marrow failure syndrome of childhood manifested as macrocytic anemia with insufficient erythroid precursors in the bone marrow. Within the decade following the demonstration that mutations in the ribosomal protein gene *RPS19* can lead to DBA, this disease has become a paradigm for an emerging group of pathologies (ribosomopathies) linked to defects in ribosome biogenesis. Mutations in ribosomal protein genes impair ribosome biogenesis, resulting in activation of the p53 tumor suppressor pathway, cell cycle arrest and defective erythropoiesis. While mutations in ribosomal protein genes have been found in 50-60% of DBA patients, genetic abnormalities in the remaining patients are largely unknown. Despite improvements in our understanding of the pathophysiology of DBA, the molecular basis for selective impairment of the erythroid lineage in this disorder is not understood. In particular, how ribosome biogenesis is regulated in erythroid precursors remains elusive. Our laboratory has been investigating the role of Polycomb group protein Bmi1 in regulating hematopoietic stem cell (HSC) self-renewal and lineage commitment. Recently, we found that Bmi1 is a critical downstream target of AKT signaling and AKT-mediated phosphorylation of Bmi1 inhibits HSC self-renewal (Liu et al., ***Science Signaling,*** 2012). Upon more detailed analysis of the hematopoietic phenotype of the *Bmi1* knockout mice, we have observed that these mice develop macrocytic anemia and show delayed recovery following phenylhydrazine (PHZ)-induced hemolytic anemia. This phenotype suggests defective erythropoiesis and we identified that loss of *Bmi1* expression results in a block in erythroid differentiation and decreased erythroid colony formation. Gene expression profiling indicated that multiple ribosomal protein genes were downregulated in *Bmi1* null erythroid precursors. Moreover, we discovered that the p53 pathway is activated in *Bmi1* null erythroid progenitor cells and genetic inhibition of p53 activity rescued the erythroid defects in the *Bmi1* deficient mice. Thus, *Bmi1* null mice recapitulate many critical features of human DBA. Furthermore, we demonstrated that BMI1 plays a critical role in human erythropoiesis as knockdown of *BMI1* in human CD34+ cells decreases ribosomal protein gene expression, activates the p53 pathway, and blocks erythroid differentiation. Importantly, we observed that *BMI1* expression is downregulated in bone marrow cells from some DBA patients. Thus, BMI1 plays a critical role in regulating ribosome biogenesis in erythroid precursors and BMI1 deficiency may contribute to the pathogenesis of DBA. Understanding how BMI1 regulates ribosome biogenesis and erythroid development will provide novel insight into the processes by which BMI1 and ribosomopathies contribute to the pathogenesis of DBA and potentially new targets for therapeutic intervention.

PRL2 maintains hematopoietic stem and progenitor cells through regulating SCF/KIT signaling

Michihiro Kobayashi, Yuanshu Dong, Hao Yu, Yunpeng Bai, Sisi Chen, Rui Gao, Lujuan Zhang, Mervin C. Yoder, Reuben Kapur, Zhong-Yin Zhang, **Yan Liu**.

The phosphatase of regenerating liver family of phosphatases, consisting of PRL1, PRL2 and PRL3, represents an intriguing group of proteins implicated in cell proliferation and tumorigenesis. However, the role of PRLs in normal and malignant hematopoiesis is largely unknown. While SCF/KIT signaling plays an important role in hematopoietic stem and progenitor cell (HSPC) maintenance, how SCF/KIT signaling is regulated in HSPCs remains poorly understood. We here report that PRL2 regulates HSPC maintenance through regulating SCF/KIT signaling. To define the role of PRL2 in hematopoiesis, we analyzed the hematopoietic stem cell (HSC) behavior in *Prl2* deficient mice generated by our group. *Prl2* deficiency results in ineffective hematopoiesis and impairs the long-term repopulating ability of HSCs. In addition, *Prl2* null HSPCs are less proliferative and show decreased colony formation in response to SCF stimulation. Furthermore, *Prl2* null HSPCs show reduced activation of the PI3K/AKT and ERK signaling in steady state and following SCF stimulation. Importantly, we found that the ability of PRL2 to enhance SCF signaling depends on its enzymatic activity, demonstrating that PRL2 mediates SCF/KIT signaling in HSPCs. Thus, PRL2 plays a critical role in hematopoietic stem and progenitor cell maintenance through regulating SCF/KIT signaling. Furthermore, loss of *Prl2* decreased the ability of oncogenic KITD814V mutant in promoting hematopoietic progenitor cell proliferation and in activation of signaling pathways. We also checked the expression of PRL2 proteins in human AML cell lines and found increased level of PRL2 proteins in some acute myeloid leukemia (AML) cells compared with normal human bone marrow cells, indicating that PRL2 may play a pathological role in AML. Our results suggest that the PRL2 phosphatase may be a druggable target in myeloproliferative disease (MPD) and acute myeloid leukemia (AML) with oncogenic KIT mutations.

High-Risk AML, Including Flt3ITD+, Exhibits Resistance To Conventional Cytarabine Induction Associated With Diminished ENT1 and p16INK Expression: Evidence For Epigenetic Repression and HDAC Inhibitor Modulated De-Repression

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Resistance to conventional induction chemotherapy involving continuous infusion cytarabine, 100mg/m2 X 7 days, has been observed in several poor-risk AML phenotypes including normal karyotype with Flt3ITD or DNMT3A mutations (JP Patel, NEJM; 2012). However, escalated anthracycline dosing along with conventional cytarabine was determined to confer superior survival among certain subgroups, including mDNMT3A, in the randomized E1900 trial. We asked whether optimal therapy among other AML molecular subgroups, where the former combination may be inferior, might preferentially require intermediate/high-dose cytarabine within induction regimens containing conventional anthracycline. This hypothesis was generated in recognition of diminished cytosine analog (cytarabine, azacytidine) transport by the rate-limiting equilibrative nucleoside transporter (ENT1) in poor-risk groups (J Hummel-Eisenbeiss, Mol Pharm, 2013) and observation of disparate cytarabine LC50's among known AML subgroups: normal karyotype (NK), =/> 10 micromolar; CBF+ve, 1-4 micromolar. In an attempt to identify an impact of diminished ENT1 expression on successful remission induction at day+14 of conventional "7+3" therapy and/or associated severe limitation of event-free survival, an unselected group of 20 AML patients' blasts were comparatively studied for ENT1 by immunoblot and/or quantitative RT-PCR, and as well were subjected to parallel analysis of p16INK4a transcripts, whose diminution, accompanying an epigenetic mechanism, is also implicated in poor treatment outcomes (HJ DeJonge, Blood; 2009). Median follow-up of the 20 patients exceeded 12 months. Among patients with high ENT1 expression by immunoblot (50kd species) were included CBF+ve and NK AMLs, of whom 7/8 pts. overall (2 CBF+ve, 1 NK-mNPM1, 1 NK-absent Flt3ITD, 2 NK Flt3ITD+, 1 complex/trisomy 13) had no evidence of leukemia on a day +14 marrow, and the event-free survival exceeded the 12 month mark. By contrast, among patients with 2-5-fold lower blast cell ENT1 expression were included a higher fraction of poor-risk (complex) karyotype or normal karyotype with Flt3ITD mutation (62%). These patients either had persistent disease at day +14, or had less than 3-month event-free survival. There was correspondence for differing ENT1 protein expressions with transcript quantity, and coupling between low/nondetection or high/detection of both ENT1/p16INK4, respectively, predicted divergent outcome of therapy from usual prognostic groups. Because the mechanism of repressed ENT1 transcriptional expression resulting from transduction of Flt3ITD gene in model AML systems is linked to transfactor c-jun interaction on the ENT1/SLC29A1

promoter, we tested the activity of tyrosine kinase/FLT3 inhibitor or HDAC inhibitor (SAHA), or their combination, on upmodulation of ENT1 in cultured primary blasts. In patient blasts in which phospho-c-jun expression was associated with diminished ENT1 expression, application of inhibitor(s) yielded downregulation of p-jun and upregulation of ENT1. This result supports an initiative to introduce both epigenetic agent(s) along with intermediate-dose cytarabine into induction therapy of certain poor-risk AML subgroups, including Flt3ITD+.

BMI1 regulates ribosome biogenesis in erythroid progenitor cells

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Our laboratory has been investigating the role of Polycomb group protein Bmi1 in regulating hematopoietic stem cell (HSC) self-renewal and lineage commitment. Recently, we found that Bmi1 is a critical downstream target of AKT signaling and AKT-mediated phosphorylation of Bmi1 inhibits HSC self-renewal (Liu et al., ***Science Signaling,*** 2012). Upon more detailed analysis of the hematopoietic phenotype of the *Bmi1* knockout mice, we have observed that these mice develop macrocytic anemia and show delayed recovery following phenylhydrazine (PHZ)-induced hemolytic anemia. This phenotype suggests defective erythropoiesis and we identified that loss of *Bmi1* expression results in a block in erythroid differentiation and decreased erythroid colony formation. Gene expression profiling indicated that multiple ribosomal protein genes were downregulated in *Bmi1* null erythroid precursors. To verify the microarray data, we performed quantitative RT-PCR assays and observed decreased expression of multiple ribosomal protein genes encoding both the small and large subunits of ribosome, including *RPS14*, *RPS19, RPS27* and *RPL11*, which has been implicated in the pathogenesis of diamond blackfan anemia (DBA). Moreover, we discovered that the p53 pathway is activated in *Bmi1* null erythroid progenitor cells and genetic inhibition of p53 activity rescued the erythroid defects in the *Bmi1* deficient mice. Furthermore, we demonstrated that BMI1 plays a critical role in human erythropoiesis as knockdown of *BMI1* in human CD34+ cells decreases ribosomal protein gene expression, activates the p53 pathway, and blocks erythroid differentiation. Thus, erythroid defects seen in Bmi1 deficient mouse and human cells recapitulate some features of human DBA.

To investigate whether BMI1 directly regulates ribosomal protein gene expression, we performed ChIP-seq experiments in a magakarycytic cell lines with Ring 1B antibody and found that PRC1 complex associates with transcription start site of several ribosomal protein genes. To confirm the ChIP-seq data, we performed ChIP experiments and found that both BMI1 and Ring1B bind to promoters of multiple ribosomal protein genes, including *RPS14*, *RPS19* and RPL 11 in K562 cells. Moreover, we found that overexpress BMI1 activates RPL11 promoter,

whereas knockdown of BMi1 decreases RPL11 promoter activation. These data demonstrate that BMI1 associates with the promoter of ribosomal protein genes and positively regulate their transcription in erythroid cells. Given that RPS19 is downregulated in the absence of BMI1, we reasoned that overexpression of BMI1 may rescue the erythroid defects triggered by RPS19 deficiency. This is indeed the case. While ectopic BMI1 expression did not increased the colony number of human cord blood CD34+ cells expressing RPS19 shRNA, it significantly increased the colony size compared with control shRNA transduced cells. Thus, ectopic BMI1 expression partially rescues the proliferation defects induced by RPS19 deficiency. Similar experiments with primary cells from DBA patient are ongoing. We predict that ectopic BMI1 expression can rescue the erythroid defects seen in DBA. Thus, BMI1 plays a critical role in regulating ribosome biogenesis in erythroid precursors and BMI1 deficiency may contribute to the pathogenesis of DBA.

PRL2 phosphatase mediates oncogenic KIT and FLT3 signaling in acute myeloid leukemia

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The phosphatase of regenerating liver (PRL) family of phosphatases, consisting of PRL1, PRL2, and PRL3, represents an intriguing group of proteins being validated as therapeutic targets in solid tumors. While PRL2 is overexpressed in human acute myeloid leukemia (AML), its role in normal and malignant hematopoiesis is largely unknown. Recently, we reported that PRL2 regulates hematopoietic stem cell self-renewal through regulating SCF/KIT signaling (Kobayashi et al., Stem Cells, 2014). To define the role of PRL2 in the pathogenesis of AML, we overexpressed KIT-D814V or FLT3-ITD in *Prl2* null hematopoietic progenitor cells and performed *in vitro* and *in vivo* assays. We discovered that *Prl2* null Lin^- cells expressing KITD814V or FLT3-ITD mutant show decreased proliferation compared with wild-type cells in the absence of cytokine. Moreover, PRL2 deficiency significantly delayed the leukemogenesis induced by KITD814V *in vivo*, demonstrating that PRL2 is important for KITD814V and FLT3-ITD-mediated ligand-independent growth of hematopoietic progenitor cells *in vitro* and leukemogenesis *in vivo*. Furthermore, we observed increased level of PRL2 proteins in some AML cell lines and inhibition of PRL2 activity with a PRL2-specific small molecule inhibitor (PRLi) results in decreased proliferation and apoptotic cell death of human AML cell lines expressing PRL2. Importantly, we found that primary human AML cells isolated from patients with FLT3-ITD positive AML and normal karyotype AML are sensitive to PRL2 inhibitor treatment in a dosage-dependent manner, suggesting that human AML cells are dependent on PRL2 activity for proliferation and survival. PRL2 is a dual specificity protein phosphatase. However, the substrates of PRL2 are largely unknown. To identify novel targets of PRL2 activity in AML, we performed protein phosphatase substrate trap assays and identified several potential PRL2 substrates in Kasumi cells, including KIT, SHP2, CBL and PLC-γ. We found that the levels of pAKT and pERK1/2 are significantly lower in *Prl2* null Kit^+ cells compared with those in wild-type cells. When we overexpressed wild-type or the catalytic inactive mutant form of PRL2 (PRL2/C101S) in Lin^- cells isolated from *Prl2* null mice, we found that wild-type PRL2, but not the mutant PRL2, augments pAKT and pERK1/2 levels in *Prl2* null Lin^- cells, indicating that the ability of PRL2 to enhance cytokine signaling in hematopoietic cells depends on its phosphatase activity. Mechanistically, PRL2 deficiency results in decreased KIT stability and phosphorylation following SCF stimulation, possibly through dephosphorylating CBL. Thus, PRL2 phosphatase is an important mediator of oncogenic signaling in AML and may be a druggable target in myeloproliferative disease (MPD) and acute myeloid leukemia (AML) with oncogenic KIT and FLT3 mutations.

PRL2 phosphatase mediates oncogenic KIT and FLT3 signaling in acute myeloid leukemia

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The phosphatase of regenerating liver (PRL) family of phosphatases, consisting of PRL1, PRL2, and PRL3, represents an intriguing group of proteins being validated as therapeutic targets in solid tumors. While PRL2 is overexpressed in human acute myeloid leukemia (AML), its role in normal and malignant hematopoiesis is largely unknown. Recently, we reported that PRL2 regulates hematopoietic stem cell self-renewal through regulating SCF/KIT signaling (Kobayashi et al., Stem Cells, 2014). To define the role of PRL2 in the pathogenesis of AML, we overexpressed KIT-D814V or FLT3-ITD in *Prl2* null hematopoietic progenitor cells and performed *in vitro* and *in vivo* assays. We discovered that *Prl2* null Lin^- cells expressing KITD814V or FLT3-ITD mutant show decreased proliferation compared with wild-type cells in the absence of cytokine. Moreover, PRL2 deficiency significantly delayed the leukemogenesis induced by KITD814V *in vivo*, demonstrating that PRL2 is important for KITD814V and FLT3-ITD-mediated ligand-independent growth of hematopoietic progenitor cells *in vitro* and leukemogenesis *in vivo*. Furthermore, we observed increased level of PRL2 proteins in some AML cell lines and inhibition of PRL2 activity with a PRL2-specific small molecule inhibitor (PRLi) results in decreased proliferation and apoptotic cell death of human AML cell lines expressing PRL2. Importantly, we found that primary human AML cells isolated from patients with FLT3-ITD positive AML and normal karyotype AML are sensitive to PRL2 inhibitor treatment in a dosage-dependent manner, suggesting that human AML cells are dependent on PRL2 activity for proliferation and survival. PRL2 is a dual specificity protein phosphatase. However, the substrates of PRL2 are largely unknown. To identify novel targets of PRL2 activity in AML, we performed protein phosphatase substrate trap assays and identified several potential PRL2 substrates in Kasumi cells, including KIT, SHP2, CBL and PLC-γ. We found that the levels of pAKT and pERK1/2 are significantly lower in *Prl2* null Kit^+ cells compared with those in wild-type cells. When we overexpressed wild-type or the catalytic inactive mutant form of PRL2 (PRL2/C101S) in Lin^- cells isolated from *Prl2* null mice, we found that wild-type PRL2, but not the mutant PRL2, augments pAKT and pERK1/2 levels in *Prl2* null Lin^- cells, indicating that the ability of PRL2 to enhance cytokine signaling in hematopoietic cells depends on its phosphatase activity. Mechanistically, PRL2 deficiency results in decreased KIT stability and phosphorylation following SCF stimulation, possibly through dephosphorylating CBL. Thus, PRL2 phosphatase is an important mediator of oncogenic signaling in AML and may be a druggable target in myeloproliferative disease (MPD) and acute myeloid leukemia (AML) with oncogenic KIT and FLT3 mutations.

PRL2 phosphatase regulates early stage T-cell development through fine-tuning SCF/KIT signaling

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The phosphatase of regenerating liver (PRL) family of phosphatases, consisting of PRL1, PRL2, and PRL3, represents an intriguing group of proteins being validated as biomarkers and therapeutic targets in human cancer. We have been investigating the role of PRL2 in normal and malignant hematopoiesis and found that PRL2 is important for HSC self-renewal (Kobayashi et al., Stem Cells, 2014). The receptor tyrosine kinase KIT can balance quiescence for HSC maintenance and proliferation for progeny supply. The defects seen in the PRL2-deficient hematopoietic and testis cells recapitulate the phenotype of c-Kit mutant mice, suggesting that the SCF/KIT signaling may be impaired in the absence of PRL2 (Kobayashi et al., Stem Cells, 2014; Dong et al., JBC, 2013). Given that KIT also plays critical role in maintaining postnatal T-lymphopoiesis in thymus, we hypothesized that PRL2 is important for T cell development.

Here we report that loss of PRL2 impairs T-lymphopoiesis both *in vitro* and *in vivo*. PRL2 deficiency resulted in marked reduction of splenocyte and thymocyte counts compared to wild type mice. While we observed modest increase in the frequency of early T cell progenitor (ETP), DN2, and DN3 cells in PRL2 deficient thymus, T-cell reconstitution was dramatically decreased after HSC transplantation. T-cell number in the peripheral blood (PB) of recipient mice populated with PRL2-null HSCs was 30 times less than that of the wild type HSCs (WT: 2288.6±579.8 /μl vs PRL2 null: 69.5±22.1 /μl, $p<0.00001$). Although the frequency of donor-derived thymocytes in WT recipient thymus was 91±6.1%, PRL2 null HSCs only contributed 7.1±4.9% ($p<0.00001$) in the recipient thymus. Surprisingly, chimerism in ETP was comparable between WT and PRL2 null cells (WT: 91.8±10.1% vs PRL2 null: 59.6±13.5%, $p<0.01$). Importantly, the frequency of thymocytes derived from PRL2 null HSCs fell down to 10% in DN2, whereas WT HSCs consistently contributed around 90%, suggesting that the DN1-to-DN2 transition requires PRL2. Next, we evaluated the *in vitro* T-cell generation by utilizing the Delta-Like1 (DLL1) expressing OP9 (DL-OP9) stromal cells. While wild type KSLs produced massive amount of T-cells (fold increase: 33,000±3371) 22 days following plating onto the DL-OP9 cells, PRL2 null KSLs only generated limited amount of T-cells (fold increase: 1765±665, $p<0.0001$), demonstrating that PRL2 is important for T-cell proliferation. We also monitored the generation of ETPs from KSLs in DL-OP9 cultures and observed significant expansion of ETPs derived from wild type KSLs compared to that of the PRL2 null KSLs (fold increase: 183.8±14.4 vs 12.5±4.3, $p<0.001$). When sorted DN3 cells from WT and PRL2 null thymus were plated onto DL-OP9 cultures, we saw moderate increase in cell expansion, suggesting that PRL2 regulate early T-cell development.

While PRL2 is a dual specificity protein phosphatase, its substrates are largely unknown. To identify PRL2 substrates in hematopoietic cells, we performed a protein phosphatase substrate trap assay. We utilized a GST-tagged PRL2-CS/DA mutant, in which the catalytic site cysteine was mutated to serine, so that PRL2 binds to its substrates, but is unable to dephosphorylate them. We found that the mutant PRL2 (PRL2/CS-DA) shows enhanced association with KIT than wild-type PRL2 in Kasumi-1 cells, suggesting that KIT is a potential PRL2 substrate. The PRL2 and KIT interaction was further confirmed by the Immunoprecipitation (IP) assay in 293T cells expressing KIT. We also detected the

association of PRL2 with SHP2, CBL and PLC-γ in Kasumi-1 cells, which are important regulators of KIT activation and stability. Moreover, PRL2 null hematopoietic progenitor cells show decreased KIT phosphorylation at tyrosine 703 following SCF stimulation, suggesting that PRL2 may modulate KIT activation in these cells. To evaluate the impact of SCF stimulation on T-cell proliferation, we cultured lymphoprimed multipotent progenitor cells (LMPPs) purified from WT and PRL2 null mice onto DLL-Fc coated plates with increasing doses of SCF (0.2, 1, 5, 25 ng/ml). The total number of cells generated from SCF treated wild type LMPPs was significantly higher than that of the PRL2 null cells in a dosage dependent manner, indicating that PRL2 fine-tunes SCF signal intensity in early T-cell.

Taken together, we have identified a critical role for PRL2 in T-cell proliferation and maintenance through fine-tuning SCF/KIT signaling.

Gain-of-function mutant p53 enhances hematopoietic stem cell self-renewal

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The tumor suppressor p53 is a critical regulator of hematopoietic stem cell (HSC) behavior and we demonstrated that p53 maintains HSC quiescence and regulates HSC response to irradiation (Liu et al., Cell Stem Cell, 2009). While *TP53* mutations are less common in acute myeloid leukemia (5 to 8%) than in solid tumors (50%), they are associated with poor prognosis and abnormal cytogenetics, especially abnormalities in chromosomes 5 and 7. These mutations may abolish some but not necessarily all of the functions of p53 in regulating stem cell behavior. Therefore, this aspect of p53 function needs further investigation.

To define the role of mutant p53 in the pathogenesis of AML, we introduced 9 hot-spot p53 mutants identified in AML patients, including $p53^{R248W}$, $p53^{R273H}$ and $p53^{Y220C}$, into wild type hematopoietic cells using retrovirus-mediated transduction and investigated the role of these p53 mutants in regulating HSC self-renewal. We found that hematopoietic cells expressing $p53^{R248W}$, $p53^{Y220C}$ or $p53^{R273H}$ show enhanced repopulating potential 16 weeks following transplantation. As codon 248 of the p53 protein is most frequently mutated in AML, we decided to investigate the role of $p53^{R248W}$ mutant in HSCs by using the humanized knock-in mice of $p53^{R248W}$. In p53 knockout mice, there is a dramatic increase of HSCs (CD48-CD150+Lin-Sca1+c-Kit+ cells); however, we found that both wild type and $p53^{R248W}$ mice have similar number of HSCs. While wild type p53 maintains HSC quiescence, expression of $p53^{R248W}$ in HSCs (CD48-CD150+LSKs) does not affect their quiescent state. As $p53^{R248W}$ does not appear to affect HSC frequency and quiescence, it is not a loss-of-function mutant. We also used bone marrow cells isolated from both wild type and $p53^{R248W}$ mice to perform the serial replating assays and found that expressing $p53^{R248W}$ from the endogenous *Trp53* promoter enhances the replating potential of hematopoietic cells**.** Moreover, we performed serial bone marrow repopulation (BMT) assays and found that the repopulating ability of $p53^{R248W}$ cells was significantly higher than that of the wild type cells in both primary and secondary BMT assays, demonstrating that the $p53^{R248W}$ mutant enhances HSC self-renewal in vivo. Furthermore, we observed that HSCs expressing $p53^{R248W}$ are resistant to genotoxic stress induced by irradiation and the $p53^{R248W}$ mice show extended survival following sub-lethal dose of total body irradiation. Ample data indicate that mutant p53 proteins not only lose their tumor suppressive functions, but also gain new abilities that promote tumorigenesis. To understand how mutant p53 enhances HSC self-renewal, we performed gene expression profiling assays by using HSCs isolated from wild type and $p53^{R248W}$ mice. We also utilized Ingenuity Pathway analysis software to group putative mutant p53 target genes into different pathways. While we did not observe change in the expression of p53 target genes in $p53^{R248W}$ HSCs, several pathways that are important for leukemogenesis, including epigenetic and DNA damage repair pathways, are altered in HSCs expressing $p53^{R248W}$, demonstrating that $p53^{R248W}$ is a gain-of-function mutant. Given that *TP53* mutations are correlated with poor prognosis, pharmacological inhibition of mutant p53 may be a promising therapeutic strategy for AML patients with *TP53* mutations. Small molecule PRIMA-1 has been shown to restore wild-type conformation to some mutant p53 proteins and induce apoptosis in human tumor cells. We found that hematopoietic cells expressing mutant p53 are sensitive to PRIMA-1 treatment and undergo p53-dependent apoptosis. Furthermore, we observed that PRIMA-1 inhibits the growth of primary human AML cells with *TP53* mutation in a dosage-dependent manner.

Taken together, we demonstrated that gain-of-function mutant p53 enhances hematopoietic stem cell self-renewal through regulating epigenetic and DNA damage repair pathways. Our data also suggest that pharmacological inhibition of mutant p53 may sensitize the drug-resistant leukemia stem cells (LSCs) to chemotherapy and improves leukemia treatment.

Gain-of-function mutant p53 enhances hematopoietic stem cell self-renewal

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The tumor suppressor p53 is a critical regulator of hematopoietic stem cell (HSC) behavior and we demonstrated that p53 maintains HSC quiescence and regulates HSC response to irradiation (Liu et al., Cell Stem Cell, 2009). While *TP53* mutations are less common in acute myeloid leukemia (10 to 15%) than in solid tumors (50%), they are associated with poor prognosis and abnormal cytogenetics, especially abnormalities in chromosomes 5 and 7. To define the role of mutant p53 in the pathogenesis of AML, we introduced 9 hot-spot p53 mutants identified in AML patients, including $p53^{R248W}$, $p53^{R273H}$ and $p53^{Y220C}$, into wild type hematopoietic cells using retrovirus-mediated transduction and investigated the role of these p53 mutants in regulating HSC self-renewal. We found that hematopoietic cells expressing $p53^{R248W}$, $p53^{Y220C}$ or $p53^{R273H}$ show enhanced repopulating potential 16 weeks following transplantation. As codon 248 of the p53 protein is most frequently mutated in AML, we decided to investigate the role of $p53^{R248W}$ mutant in HSCs by using the humanized knock-in mice of $p53^{R248W}$. In p53 knockout mice, there is a dramatic increase of HSCs; however, we found that both wild type and $p53^{R248W}$ mice have similar number of HSCs. While wild type p53 maintains HSC quiescence, expression of $p53^{R248W}$ in HSCs does not affect their quiescent state. As $p53^{R248W}$ does not appear to affect HSC frequency and quiescence, it is not a loss-of-function mutant. We also used bone marrow cells isolated from both wild type and $p53^{R248W}$ mice to perform the serial replating assays and found that expressing $p53^{R248W}$ from the endogenous *Trp53* promoter enhances the replating potential of hematopoietic cells**.** Moreover, we performed serial bone marrow repopulation (BMT) assays and found that the repopulating ability of $p53^{R248W}$ cells was significantly higher than that of the wild type cells in both primary and secondary BMT assays, demonstrating that the $p53^{R248W}$ mutant enhances HSC self-renewal in vivo. Furthermore, we observed that HSCs expressing $p53^{R248W}$ are resistant to genotoxic stress induced by irradiation and the $p53^{R248W}$ mice show extended survival following sub-lethal dose of total body irradiation. Ample data indicate that mutant p53 proteins not only lose their tumor suppressive functions, but also gain new abilities that promote tumorigenesis. To understand how mutant p53 enhances HSC self-renewal, we performed gene expression profiling assays by using HSCs isolated from wild type and $p53^{R248W}$ mice. We also utilized Ingenuity Pathway analysis software to group putative mutant p53 target genes into different pathways. While we did not observe change in the expression of p53 target genes in $p53^{R248W}$ HSCs, several pathways that are important for leukemogenesis, including epigenetic and DNA damage repair pathways, are altered in HSCs expressing $p53^{R248W}$, demonstrating that $p53^{R248W}$ is a gain-of-function mutant. Taken together, we demonstrated that gain-of-function mutant p53 enhances hematopoietic stem cell self-renewal through regulating epigenetic and DNA damage repair pathways.

Cyclic Administration of Epigenetically-active Agents, acting in Id1-RUNX3-TET2 as well as hoxA9/10 pathways, in Poor-Risk AML: Consecutive Phase I Trials Identify Therapeutic Superiority Involving Bortezomib in Flt3ITD+ AML. H Sayar, HS Boswell, R Gao, M AbuZaid, LD Cripe, AM Wilson-Weekes, M Nassiri, L Li, S Gupta, A Suvannasankha, F Pan, R Shanmugam, C Goswami, M Xu, Y Liu

Definition of the Problem: Progress for poor-risk AML subtypes in response to conventional chemotherapy has been stagnant. Ontogenic studies of blasts present at initial disease presentation, in remission, and at relapse, demonstrate that founder clones have mutations within epigenetic modifiers that persist and contribute to relapse (Corces-Zimmerman MR, et al. 2014; PNAS. 111:2548-53). Among those mutant epigenetic modifiers is TET2 (ten-eleven translocation two). Mutations or loss of expression of TET2 results in repression of tumor suppressor genes that would otherwise allow for clonal extinction with chemotherapy. We identified within poor-risk AMLs, 3 tumor suppressor genes are repressed in tandem: RUNX3, DAPK1, P16INK4A. Among these, RUNX3 is a known protein antagonist of β-catenin, which is active in hoxA10/CDX4 transcription important for leukemic stem cell maintenance (Bei L, et al. JBC. 2012: 39589-601). Thus, RUNX3 repression may be a crucial output from TET2/WT1 loss toward augmented β-catenin targets, and may be susceptible to epigenetically active agents.

Approach: We investigated tumor suppressor genes affected in epigenetic repression by AML-signaling pathways, including Flt3ITD+*ve*, for reactivation to cellular stress/senescence by combination of tyrosine kinase inhibitor with histone deacetylase inhibitor; or with additional Bortezomib. Consecutive phase 1 clinical trials pursued cyclic administration of Sorafenib, targeting Flt3 and Raf/MAPK, along with Vorinostat, pan-HDAC inhibitor, to poor-risk/refractory AML patients. In the second trial, Bortezomib was added to these in optimal doses. A 21-day schedule with 14 days of treatment was used. Optimal dosing was Sorafenib 400 mg and Vorinostat 200 mg, both administered twice-daily, and Bortezomib was escalated to 1.3mg/m2 on days 1,4,8,11. Pharmacodynamic monitoring performed on marrow blasts before (d0) and early during therapy (d3/4) tested linkage of signaling with epigenetic targets, especially whether Flt3/stat5-induced Id1 may influence *p16INK4a* and/or *RUNX3* repression. We also asked if Flt3ITD signals to stat5/c-jun/p52NF-κB and PIM1/2 expression or DAPK1 repression affect blunted apoptosis.

Results: In the first trial, 14 patients were evaluable for response: 43% patients demonstrated PR, and 1/14 (7%) achieved a CR. Three/six (50%) PRs had Flt3ITD. In the second trial, significant enhancement of response rate was observed, specifically in the Flt3ITD+*ve* population, with CR/CRi approaching 59% by 2 cycles among 17 total patients. Responding patient blasts demonstrated early depletion of nuclear p52NF-κB and/or stat5/c-jun along with Id1, accompanied by apoptosis activation. Blast *TET2* transcript deficiency signaled enhanced activity of the combinations, associated with treatment-induced *RUNX3* de-repression. *RUNX3* de-repression was linked to severe treatment-induced diminution of hoxA9/10 expression and response. Indeed, among 15 samples across both trials available for multigene molecular analysis, relief of *RUNX3* repression occurred in 7/8 CR/CRi/VGPR (p=0.010), associated with severe *TET2* hypo-expression in 6/7, whereas 5/6 non-responders failed to demonstrate RUNX3 de-repression. *HoxA9/10* and jun expression were reduced in tandem in 7/8 (0.010) and 5/7 (NS) responders, respectively, but only in 1/7 non-responders.

Conclusions: These results confirm the existence of an interaction of Flt3ITD with *TET2* hypo-expression in AML, which creates a novel composite pathway (recently seen in a double mutant mouse model: Shih AH, et al. Cancer Cell. 2015;27:502-15.) involving severely depressed *RUNX3-TET2*. Loss of RUNX3 affects unattenuated heightened β-catenin activity, thus promoting leukemic/pre-leukemic stem cell persistence, in part through *hoxA9/10* hyperexpression. Also, c-jun is known to interact with β-catenin in expression of certain genes, which may include hoxA9/10. The results provide a roadmap for epigenetically-targetedd intervention on the RUNX3-TET2-β-catenin pathway to hoxA/CDX4 overexpression to allow reversal of chemotherapeutic resistance.

Polycomb Repressive Complex 1 enhances HSC self-renewal through repressing the canonical Wnt signaling pathway

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Polycomb group (PcG) proteins are epigenetic gene silencers that have been implicated in stem cell maintenance and cancer development. Genetic and biochemical studies indicate that Polycomb group proteins exist in at least two protein complexes, Polycomb repressive complex 2 (PRC2) and Polycomb repressive complex 1 (PRC1), that act in concert to initiate and maintain stable gene repression. While studies on individual PRC1 component suggest that PRC1 plays an important role in hematopoiesis, how PRC1 regulates transcriptional repression in hematopoietic stem cells (HSCs) is largely unknown. Bmi1 and Mel18 are two major homologs of the PCGF subunit within the PRC1 complex. Bmi1 is a positive regulator plays of HSC self-renewal; however, the role of Mel18 in hematopoiesis has been controversial. To determine whether Bmi1 and Mel18 play redundant or distinct role in HSC self-renewal, we have generated Bmi1 and Mel18 conditional knockout mice. While acute deletion of Mel18 affects neither HSC frequency nor lineage commitment, we found that Mel18-deficent hematopoietic progenitor cells showed enhanced replating potential compared to wild type cells. To determine the role of Mel18 in HSC self-renewal, we performed serial HSC transplantation assays and found that the repopulating ability of $Mel18^{-/-}$ HSCs was significantly higher than WT HSCs in both primary and secondary transplantation assays, demonstrating that the loss of Mel18 enhances HSC self-renewal *in vivo*. We hypothesize that loss of Bmi1 and Mel18 in hematopoietic stem cells will disrupt PRC1 complex and impairs HSC self-renewal. To determine the role of PRC1 complex in HSCs, we analyzed the HSC behavior in Bmi1 and Mel18 double-deficient mice. While we found that Bmi1-deficient HSCs showed decreased repopulating potential compared to WT HSCs 16 weeks following transplantation, loss of both Bmi1 and Mel18 in HSCs resulted in even more severe self-renewal defects. In addition, loss of both Bmi1 and Mel18 resulted in decreased myeloid differentiation and increased B cell differentiation compared to WT, Mel18, and Bmi1 KO mice. These data demonstrate that Bmi1 and Mel18 have non-overlapping role in HSC maintenance and lineage commitment.

Given that Bmi1 plays a dominant role in the PRC1 complex, we decided to identify Bmi1 target genes in hematopoietic stem cells to understand how PRC1 complex regulates HSC self-renewal. We performed transcript profiling assays to compare gene expression in HSCs isolated from wild type and $Bmi1^{-/-}$ mice. The Ingenuity Pathways indicates that the canonical Wnt signaling is significant elevated in Bmi1 null HSCs compared to WT HSCs. We confirmed the upregulation of several genes of the Wnt pathway in Bmi1 null HSCs by quantitative real-time PCR analysis. To determine whether Bmi1 can repress the activation of Wnt signaling in cells, we utilized the Top-Flash Wnt reporter system. Stimulation of 293T cells with Wnt3a activates the Wnt reporter and this activation can be efficiently repressed by Bmi1. Furthermore, we detected the association of Bmi1 with the Lef1, Tcf4, and Axin2 promoters in the ChIP experiment. Thus, Bmi1 directly represses the expression of several Wnt genes in hematopoietic cells. To determine the functional significance of activation of Wnt signaling in Bmi1 null HSCs, we have generated $R26Stop^{FL}$Bmi1-$Apc^{f/f}$-Mx1-Cre+ and $Bmi1^{f/f}$-$Ctnnb1^{f/f}$-Mx1-Cre+ mice. Loss of Apc in hematopoietic cells activates the Wnt signaling pathway and impairs HSC self-renewal. We found that expressing three-copies of Bmi1 from the Rosa26 locus enhanced the self-renewal capabilities of Apc deficient HSCs in transplantation assays. Ctnnb1 encodes β-catenin and loss of Ctbbn1 in HSCs diminishes Wnt signaling. Acute deletion of Bmi1 in hematopoietic compartment resulted in decreased bone marrow cellularity and enhanced apoptosis of hematopoietic stem and progenitor cells. Deletion of Ctnnb1 in Bmi1 null hematopoietic cells rescued these defects. Thus, impaired HSC self-renewal seen in Bmi1 null mice is, at least in part, due to activation of the canonical Wnt signaling pathway.

Taken together, we demonstrate that PRC1 complex enhances HSC self-renewal through inhibiting the canonical Wnt signaling.

Mutant p53 drives the Development of Pre-Leukemic Hematopoietic Stem Cells through Modulating Epigenetic Regulators

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AML is thought to arise from leukemia stem cells (LSCs); however, recent evidence suggests that the transforming events may initially give rise to pre-leukemic hematopoietic stem cells (pre-leukemic HSCs), preceding the formation of fully transformed LSCs. Pre-leukemic HSCs have been shown to contribute to normal blood development and harbor a selective growth advantage compared to normal HSCs. Pre-leukemic HSCs can acquire subsequent mutations, and once differentiation capacity is impaired, leukemia emerges. Recently, acquired somatic *TP53* mutations, including $p53^{R248W}$ and $p53^{R273H}$, were identified in healthy individuals as well as AML patients, suggesting that *TP53* mutations may be early events in the pathogenesis of AML. We found that $p53^{R248W}$ HSCs showed a multi-lineage repopulation advantage over WT HSCs in transplantation experiments, demonstrating that mutant p53 confers a pre-leukemic phenotype in murine HSCs.

Although *TP53* mutations are limited in AML, *TP53* mutations do co-exist with mutations of epigenetic regulator, ASXL-1, or receptor tyrosine kinase, FLT3, in AML. Mutations in Asxl-1 are present in ~10-30% of patients with myeloid malignancies and confer poor prognosis. Loss of Asxl-1 in the hematopoietic compartment leads to a myelodysplastic-like syndrome in mice and reduced stem cell self-renewal. Internal tandem duplications in Flt3 (Flt3-ITD) occur in ~30% of AML patients and are associated with adverse clinical outcome. Flt3-ITD-positive mice develop a myeloproliferative neoplasm (MPN) and HSCs expressing Flt3-ITD have decreased self-renewal capabilities. We hypothesize that mutant p53 drives the development of pre-leukemic HSCs with enhanced self-renewal capability, allowing clonal expansion and subsequent acquisition of Asxl-1 or Flt3 mutations leading to the formation of fully transformed leukemia stem cells. To define the role of mutant p53 in $Asxl\text{-}1^{+/-}$ HSCs, we generated $p53^{R248W/+}$ $Asxl\text{-}1^{+/-}$ mice and performed *in vitro* serial replating assays as well as *in vivo* competitive bone marrow transplantation experiments. We found that $p53^{R248W}$ significantly enhanced the serial replating ability of Asxl-1-deficient bone marrow cells. Interestingly, while bone marrow from $Asxl\text{-}1^{+/-}$ mice had very poor engraftment compared to wild type bone marrow cells 16 weeks post-transplantation, the expression of $p53^{R248W}$ in $Asxl\text{-}1^{+/-}$ bone marrow rescued the defect. To examine the role of mutant p53 in Flt3-ITD-positive HSCs, we generated $p53^{R248W/+}Flt3^{ITD/+}$ mice. We found that $p53^{R248W}$ enhanced the replating ability of $Flt3^{ITD/+}$ bone marrow cells. Despite the fact that $Flt3^{ITD/+}$ bone marrow cells displayed decreased repopulating ability compared to wild type cells 16 weeks post-transplantation, expression of $p53^{R248W}$ in $Flt3^{ITD/+}$ cells rescued the defect. We are monitoring leukemia development in primary and secondary transplant recipients as well as in de novo $p53^{R248W/+}Asxl\text{-}1^{+/-}$ and $p53^{R248W/+}Flt3^{ITD/+}$ animals and predict that mutant p53 may cooperate with Asxl-1 deficiency or Flt3-ITD in the formation of LSCs to accelerate leukemia development in Asxl-1 deficient or Flt-ITD-positive neoplasms.

Mechanistically, dysregulated epigenetic control underlies the pathogenesis of AML and we discovered that mutant p53 regulates epigenetic regulators, including *Ezh1*, *Ezh2*, *Kdm2a,* and *Setd2*, in HSCs. H3K27me3 is catalyzed by EZH1 or EZH2 of the Polycomb repressing complex 2 (PRC2). Both Ezh1 and Ezh2 are important for HSC self-renewal. SETD2 is a histone H3K36 methyltransferase and mutations in *SETD2* have been identified in 6% of patients with AML. SETD2 deficiency resulted in a global loss of H3K36me3 and increased self-renewal capability of leukemia stem cells. We found that there were increased levels of H3K27me3 and decreased levels of H3K36me3 in $p53^{R248W/+}$ HSCs compared to that of the WT HSCs. In ChIP experiments, we found that $p53^{R248W}$, but not WT p53, was associated with the promoter region of *Ezh2* in mouse myeloid progenitor cells, suggesting that $p53^{R248W}$ may directly activate *Ezh2* expression in hematopoietic cells. Asxl-1 has also been shown to regulate *Ezh2*, therefore, we will investigate the disruption of epigenetic regulators in $p53^{R248W/+}Asxl\text{-}1^{+/-}$ HSCs in order to better define the molecular mechanisms responsible for their synergism. Overall, we demonstrate that mutant p53 promotes the development of pre-leukemic HSCs by a novel mechanism involving dysregulation of the epigenetic pathways.

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2012 Showalter Research Trust Fund Award

2013 Department of Defense (DoD) Career Development Award

2013 American Cancer Society (ACS) New Investigator Award

2014 Elsa U. Pardee Foundation New Investigator Award

2014 St. Baldrick's Foundation Scholar Award

2014 Alex's Lemonade Stand Foundation Research Award

2013 to present Member, NYSTEM Study Section, New York State Department of Health and the Empire State Stem Cell Board

2014 to present	Member, the Early Career Reviewer (ECR) program at the Center for Scientific Review (CSR), National Institutes of Health
2014 to present	Foreign Expert (Member), Hematology Study Section, National Natural Science Foundation of China (NSFC), China.
2014 to present	Member, Career Development Award (CDA) Program, International Human Frontier Science Program Organization (HFSP), France
2015 to present	Member, Career Development Award (CDA) Program, St. Baldrick's Foundation, USA

PROFESSIONAL DEVELOPMENT:

2011	"Nine Golden Rules" to Succeed in Research and Scholarship, provided by the Office of the Vice Chancellor for Research, IUPUI
2011 to 2012	Leadership in Academic Medicine Program (LAMP), provided by IUSM
2012	Scientific Writing from the Reader's Perspective, by Dr. George Gopen, Professor of the Practice of Rhetoric at the Duke University, sponsored by IUSM.
2012	Writing Winning Grants, By John Robertson, Ph.D., Grant Writers' Seminars and Workshops, LLC, sponsored by IUSM.
2013	Writing and Designing NIH Proposals, by Dr. Ronald Sakaguchi, Professor and Academic Director in the School of Medicine at Oregon Health & Science University, sponsored by IUSM.
2013	Writing and Designing Department of Defense (DoD) Proposals, by Cornerstone Government Affairs, sponsored by IUSM.
2013	Animal Welfare Regulations, the IACUC, and the Researcher, by the Institute Animal Care and Use Committee (IACUC) of IUSM
2013	10th International Workshop on Molecular Aspects of Stem Cell Development and Leukemia. Cincinnati, OH
2014	11th International Workshop on Molecular Aspects of Stem Cell Development and Leukemia. Cincinnati, OH

TEACHING ASSIGNMENTS:

UNDERGRADUATE SUMMER STUDENTS AND MEDICAL STUDENTS:

2007	Youyang Yang, Summer Student, Harvard University
2008-2009	Narae Bae, Graduate Student, Cornell University

2011 Joel Pollack, Undergraduate Student, Wells Center Summer Intern program, Boston University

2012 Sisi Chen, Graduate Student, Indiana University School of Medicine

2012 Zhixiang Zhang, Undergraduate Student, Wells Center Summer Intern program, Indiana University

2012 Awahngie Akwo, Medical Student, SRPAM program, Indiana University

2013 Levi Hadley, Undergraduate Student, Wells Center Summer Intern program, Indiana University

2013 Hyewon Hwang, Undergraduate Student, CEMH Summer Intern Program, Inje University, Korea

2014 Wenjing Cai, Undergraduate Student, Indiana University

2014 Chen Mu, Medical Student, SRPAM program, Indiana University

2015 Taylor Twiggs, Medical Student, SRPAM program, Indiana University

2015 Christine Wang, Medical Student, Wells Center Summer Intern program, Indiana University

GRADUATE SCHOOL THESIS COMMITTEES:

2011-2012 Wenjuan Liao, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM

Ph.D. THESIS DIRECTOR:

2012-present Sisi Chen, PhD Candidate, Department of Biochemistry and Molecular Biology, IUSM

POSTDOCTORAL FELLOWS:

2003-2006 Jinjuan Yao, MD & PhD, Postdoctoral Fellow, MSKCC

2004-2005 Yasuhiko Miyata, MD & PhD, Postdoctoral Fellow, MSKCC

2005-2006 Goro Sashida, MD & PhD, Postdoctoral Fellow, MSKCC

2007-2008 Fabiana Perna, MD, Postdoctoral Fellow, MSKCC

2008-2010 Takashi Asai, MD & PhD, Postdoctoral Fellow, MSKCC

2011-2013 Hao Yu, PhD, Postdoctoral Fellow, Indiana University School of Medicine

2011-present Michihiro Kobayashi, MD & PhD, Postdoctoral Fellow, Indiana University School of Medicine

2011-2015 Rui Gao, PhD, Postdoctoral Fellow, Indiana University School of Medicine

2014-present Sarah Nabinger, PhD, Postdoctoral Fellow, Indiana University School of Medicine

2015-present Chonghua Yao, PhD, Postdoctoral Fellow, Indiana University School of Medicine

AWARDS TO STUDENTS AND POSTDOCTORAL FELLOWS:

Abstract Achievement Award	Michihiro Kobayashi, MD, PhD, Postdoctoral Fellow; Mentor, Yan Liu, PhD, the 55th American Society of Hematology (ASH) Conference, New Orleans, LA, 2013.
Abstract Achievement Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, the 56th American Society of Hematology (ASH) Conference, San Francisco, CA, 2014.
Graduate Student Travel Award	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, IUPUI, 2014.
Honorary Mention	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, Biochemistry Research Day, IUSM, 2014.
Honorary Mention	Sisi Chen, Graduate Student; Mentor, Yan Liu, PhD, Indiana University Cancer Research Day, 2015.
Oral presentation First Prize	Chen Mu, Medical Student; Mentor, Yan Liu, PhD, the Summer Research Program in Academic Medicine (SRPAM), Indiana University, 2014.
Oral presentation First Prize	Taylor Twiggs, Medical Student; Mentor, Yan Liu, PhD, the Summer Research Program in Academic Medicine (SRPAM), Indiana University, 2015.
Oral presentation Second Prize	Christine Wang, Medical Student; Mentor, Yan Liu, PhD, Wells Center Summer Intern program, Indiana University, 2015.

GRADUATE TEACHING ASSIGNMENTS:

Developmental Genetics-G726 Spring 2012, Lecture 8: Hematopoiesis
Role: Instructor

Developmental Genetics-G726 Spring 2013, Lecture 8: Hematopoiesis
Role: Instructor

Clinical Problem Soving-X604 Spring 2013, Cases: 5-8
Role: Instructor

Clinical Problem Soving-X604 Spring 2014, Cases: 5-8
Role: Instructor

Clinical Problem Soving-X604 Spring 2015, Cases: 5-8
Role: Instructor

Stem Cell Biology-G720 Spring 2015, Lecture 8: Cancer Stem Cells
Role: Instructor

PROFESSIONAL SERVICE:

EDITORIAL BOARD:

2014 to present	International Journal of Neonatal Science and Therapeutics
2014 to present	Journal of Stem Cell Research and Transplantation
2014 to present	Stem Cells Research, Development and Therapy
2014 to present	Journal of Stem Cells and Translational Investigation
2014 to present	Pediatrics and Neonatal Biology

REVIEWER FOR JOURNALS:

2011 to present	PLoS ONE
2012 to present	Cell Stem Cell
2012 to present	Frontiers in Oncology
2012 to present	BMC Cell Biology
2012 to present	Cancer Biotherapy & Radiopharmaceutics
2013 to present	In Vitro Cellular & Developmental Biology
2013 to present	Journal of Leukocyte Biology
2013 to present	Molecular and Cellular Biochemistry
2013 to present	Tumor Biology
2014 to present	Journal of Hematology and Oncology Research
2014 to present	International Journal of Biochemistry and Molecular Biology
2014 to present	Leukemia

2014 to present Stem Cells

2015 to present PNAS

CONTRIBUTIONS TO NATIONAL/INTERNATIONAL PROFESSIONAL ORGANIZATIONS:

2013 to present Member, NYSTEM Study Section, New York State Department of Health and the Empire State Stem Cell Board

2014 to present Member, the Early Career Reviewer (ECR) program at the Center for Scientific Review (CSR), National Institutes of Health

2014 to present Foreign Expert (Member), Hematology Study Section, National Natural Science Foundation of China (NSFC), China.

2014 to present Member, Career Development Award Study Section, International Human Frontier Science Program Organization (HFSP), France

2015 to present Member, Cancer Biology Study Section, St. Baldrick's Foundation

2014 Abstract reviewer, The 56th Annual Meeting of the American Society of Hematology (ASH)

2014 Session Chair/Modulator, the session of Malignant Stem and Progenitor Cells, The 56th Annual Meeting of the American Society of Hematology (ASH)

2015 Abstract reviewer, The 57th Annual Meeting of the American Society of Hematology (ASH)

2015 Organizer, Midwest Blood Club Conference 2015.

UNIVERSITY SERVICE:

INSTITUIONAL COMMITTEE:

2012 to present Member, Institutional Animal Care and Use Committee (IACUC), Indiana University School of Medicine

REVIEWER FOR GRANTS:

2011 IUSCC ITRAC pilot project

2012 Simon Clinical Research pilot project

2013 IUSCC Pancreatic Center pilot project

2013 IUSCC Bioinformatics Core Pilot projects

2013-2015 IUSM Wright Scholarship

OTHER SERVICE:

2012 to present	Associate Editor, the Wells Center Newsletter
2012	Poster Judge, Research Day, Department of Biochemistry and Molecular Biology, March 2012
2013	Poster Judge, Cancer Research Day, Indiana University Cancer Research Day, May 2013
2013	Panelist and Judge, Wells Center Summer Intern Program, Oral Presentation, August 2013
2013	Poster Judge, Student Research Program in Academic Medicine 2013 Poster Presentations, September 2013
2013	Interview prospective international graduate students for the IBMG program, IUSM, March 2013
2014	Career Panelist, Molecular Medicine in Action (MMIA) program, IUSM, March 2014
2014	Poster Judge, Cancer Research Day, Indiana University Cancer Research Day, May 2014
2014	Panelist and Judge, Wells Center Summer Intern Program, Oral Presentation, August 2014
2014	Poster Judge, Student Research Program in Academic Medicine 2014 Poster Presentations, September 2014
2015	Career Panelist, Molecular Medicine in Action (MMIA) program, IUSM, March 2015
2015	Poster Judge, Cancer Research Day, Indiana University Cancer Research Day, May 2015
2015	Panelist and Judge, Wells Center Summer Intern Program, Oral Presentation, August 2015
2015	Poster Judge, Student Research Program in Academic Medicine 2015 Poster Presentations, September 2015

OTHER PROFESSIONAL ACTIVITIES:

INVITED LECTURES/SEMINARS:

Local

2010	Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. Department of Pediatrics, Indiana University School of Medicine, Indianapolis, IN.

2011 Molecular basis of hematopoietic stem cell quiescence and self-renewal. Department of Biochemistry and Molecular Biology seminar, Indiana University, Indianapolis, IN

2012 Molecular basis of hematopoietic stem cell self-renewal and leukemogenesis. Neonatology morning conference, Department of Pediatrics, Indiana University.

2013 Bmi1 regulates hematopoietic stem cell self-renewal and lineage commitment. Center of Excellence of Molecular Hematology, Indiana University

Regional

2011 Akt-mediated phosphorylation of Bmi1 regulates hematopoietic stem cell self-renewal. Midwest Blood Club, Cincinnati, Ohio

2012 Akt-mediated phosphorylation of Bmi1 inhibits hematopoietic stem cell self-renewal. Midwest Blood Club, Indianapolis, Indiana

2013 PRL2 maintains hematopoietic stem and progenitor cells through regulating SCF/KIT signaling. Midwest Blood Club, Cincinnati, Ohio

2015 Polycomb Repressive Complex 1 enhances HSC self-renewal through repressing the canonical Wnt signaling pathway. Midwest Blood Club, French Lick, Indiana

National

2007 Transcriptional regulation of the Normal and Leukemic Hematopoiesis. Sloan Kettering Institute Scientific Colloquium, New York, NY.

2007 Regulation of Hematopoietic Stem Cell Quiescence - a Novel Role for p53. The 49th American Society of Hematology (ASH) Conference, Atlanta, GA.

2009 Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. Children's Hospital Oakland Research Institute, Oakland, CA.

2010 Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. Rochester University School of Medicine, Rochester, NY.

2010 Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. New York University School of Medicine. New York, NY.

2010 Molecular Basis of Hematopoietic Stem Cell Quiescence and Self Renewal. St. Jude Children's Research Hospital

2013 Bmi1 regulates ribosome biogenesis during erythroid differentiation. The 55th American Society of Hematology (ASH) Conference, New Orleans, LA.

2015 Gain-of-function Mutant p53 enhances HSC self-renewal. Keystone Symposia, Hematopoiesis (B6), Keystone, CO.

2015 Polycomb Repressive Complex 1 enhances HSC self-renewal through repressing the canonical Wnt signaling pathway. The 57th American Society of Hematology (ASH) Conference, Orlando, FL.

International

2009 Using cord blood to identify cell cycle regulatory targets for treating leukemia. The 7th Annual International Umbilical Cord Blood Transplantation Symposium, Los Angeles.

2009 Necdin regulates hematopoietic stem cell quiescence. The 7th Annual International Society for Stem Cell Research (ISSCR), Barcelona, Spain.

2011 Molecular basis of hematopoietic stem cell quiescence and self-renewal. Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences, Tianjin, China

2012 Molecular basis of hematopoietic stem cell self-renewal and leukemogenesis. National Institute of Biological Sciences, Beijing, China

2012 Molecular basis of hematopoietic stem cell self-renewal and leukemogenesis. Tsinghua University, Beijing, China

2014 PRL2 phosphatase in normal and malignant hematopoiesis. Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences, Tianjin, China

2014 Polycomb group protein Bmi1 regulates hematopoietic stem cell self-renewal and differentiation. National Institute of Biological Sciences, Beijing, China

PATENTS: N/A

GRANTS AND FELLOWSHIPS:

CURRENT ACTIVE SUPPORT:

St. Baldrick's Foundation Scholar Award (PI: Liu) 07/01/14 – 06/30/17 *7.2 Calendar*
St. Baldrick's Foundation
Targeting PRL2 phosphatase in T cell acute lymphoblastic leukemia
Role: Principal Investigator

Alex's Lemonade Stand Foundation Grant (PI: Liu) 09/02/14 – 08/31/15 *0.6 Calendar*
Alex's Lemonade Stand Foundation
Targeting PRL2 phosphatase in Pediatric acute myeloid leukemia
Role: Principal Investigator

R56AI110831 (PI: Yoshimoto) 3/10-15-2/29/16 *1.2 Calendar*
NIH/NIAID
Embryonic origin and Self-renewal of B-1a cells
The goal of this study is to identify the origin of B1 cells during development and investigate the mechanisms of B-a cell self-renewal.
Role: Co-Investigator

IUSCC Pilot Project (PI: Liu) 05/01/15 – 04/30/16
Indiana University Simon Cancer Center (IUSCC)
Targeting mutant p53 to improve leukemia treatment
Role: Principal Investigator

Wells Center Seed Fund Grant (PI: Liu) 06/01/15 – 12/31/15
Wells Center for Pediatric Research
PRL2 phosphatase as a novel therapeutic target in Acute Myeloid Leukemia
Role: Principal Investigator

CEMH Pilot Project Grant (PI: Kobayashi) 11/01/14 – 10/31/15
Indiana University Center of Excellence in Molecular Hematology (CEMH)
The role of PRL2 Phosphatase in T cell development
Role: PI/Mentor

PENDING SUPPORT:

1R01AG052501-01(PI: Liu) 02/01/16 – 01/31/20 *2.4 Calendar*
NIH/NIA
Mutant p53 rejuvenates aged stem cells through modulating epigenetic regulators
Role: Principal Investigator

Children's Leukemia Research Association Grant (PI: Liu) 01/01/16 – 12/31/16
Children's Leukemia Research Association $30,000
Targeting PRL2 in acute myeloid leukemia
Role: Principal Investigator

COMPLETED SUPPORT:

W81XWH-13-1-0187 (PI: Liu) 07/01/13 – 06/30/15 *2.4 Calendar*
DOD PRCRP Career Development Award
Modulating Leukemia-initiating Cell Quiescence to Improve Leukemia Treatment
Role: Principal Investigator

NY-STEM Grant N09G164 (PIs: Liu and Nimer) 09/01/10 -
08/31/12 New York State Department of Health
Modulating hematopoietic stem cell quiescence to improve leukemia treatment
Role: Co- Principal Investigator

Biomedical Research Grant (PI: Liu) 03/1/11 - 02/28/12
Indiana University School of Medicine (IUSM)
The role of Bmi1 in hematopoietic stem cell self-
renewal Role: Principal Investigator

IUSCC ITRAC Pilot Project (PI: Liu) 07/01/11 – 12/31/12
Indiana University Simon Cancer Center (IUSCC)
Targeting mutant p53 to improve leukemia treatment
Role: Principal Investigator

CEMH Pilot Project Grant (PI: Liu) 10/01/11 – 09/30/12
Indiana University Center of Excellence in Molecular Hematology (CEMH)
The role of mutant p53 in hematopoietic stem cell self-renewal
Role: Principal Investigator

IUPUI EMPOWER Award (PI: Liu) 10/13/11 to 10/12/13
Indiana University-Purdue University Indianapolis
Enhanced Mentoring Program with Opportunities for Ways to Excel in Research
Role: Principal Investigator

IUPUI International Development Fund (IDF) Award (PI: Liu) 01/01/12 to 12/31/12 Indiana University-Purdue University Indianapolis
Targeted Therapy in Myeloid Leukemia
Role: Principal Investigator

Showalter Research Trust Fund (PI: Liu) 07/01/12 – 06/30/13 *1.2 Calendar*
Ralph W. and Grace M. Showalter Research Trust Fund
The role of gain-of-function mutant p53 in hematopoietic stem cell self-renewal
Role: Principal Investigator

IUSCC Pilot Project for Using Next-Generation Sequencing (PI: Liu) 07/01/12 – 12/31/13
Indiana University Simon Cancer Center (IUSCC)
The role of Bmi1 in the pathogenesis of myelodysplastic syndromes
Role: Principal Investigator

Indiana CTSI P3 grant (multi-PIs) 11/01/12 – 12/31/13
Collaborative Myeloproliferative Neoplasm (MPN) and Acute Myeloid Leukemia (AML) working group: A multi-investigative Team Approach
Project #2: The role of Bmi1 in the pathogenesis of AML
Principal Investigator of Project #2: Dr. Liu

ACS Institutional Research Grant (PI: Liu) 06/01/13 – 5/31/14
American Cancer Society and Indiana University Simon Cancer Center (IUSCC) $40,000
The role of PRL2 Phosphatase in Acute Myeloid Leukemia
Role: Principal Investigator

Biomedical Research Grant (PI: Liu) 07/1/13 - 06/30/14
Indiana University School of Medicine (IUSM)
Bmi1 is a key regulator of Wnt signaling in hematopoietic stem cells
Role: Principal Investigator

IUPUI Research Support Funds Grants (RSFG) Award (PI: Liu) 07/01/13 to 06/30/14 Indiana University-Purdue University Indianapolis
The role of mutant p53 in acute myeloid leukemia
Role: Principal Investigator

Pardee Foundation Research Grant (PI: Liu) 01/01/14 – 12/31/14 *2.4 Calendar*
Elsa U. Pardee Foundation

Targeting PRL2 in Acute Myeloid Leukemia
Role: Principal Investigator
ACS Institutional Research Grant (PI: Kobayashi) 06/01/14 – 5/31/15
American Cancer Society and Indiana University Simon Cancer Center (IUSCC)
The role of PRL2 Phosphatase in the pathogenesis of T-ALL
Role: PI/Mentor

Showalter Research Trust Fund (PI: Kobayashi) 07/01/14 – 06/30/15
Ralph W. and Grace M. Showalter Research Trust Fund
Targeting PRL2 in Acute Myeloid Leukemia
Role: PI/Mentor

PRINT AND ELECTRONIC PUBLICATIONS:

I. TEACHING:

BOOK CHAPTERS AND INVITED REVIEWS

II. RESEARCH, SCHOLARSHIP, OR CREATIVE ACTIVITIES

(*REFERRED JOURNALS*: *Corresponding author)

1. **Liu Y**, Wang G, Liu J, Peng X, Xie Y, Dai J, Guo S, Zhang F. Transfer of *E. coli* gutD gene into maize and regeneration of salt-tolerant transgenic plants. *Sci China C Life Sci, Feb;* 42(1):90-95, 1999.
2. Larkins BA, Dilkes BP, Dante RA, Coelho CM, Woo YM and **Liu Y**. Investigating the hows and whys of DNA endoreduplication. *J Exp Bot,* Feb; 52(355):183-92, 2001.
3. Lacorazza HD, Yamada T, **Liu Y**, Miyata Y, Sivina M, Numes J, and Nimer SD. The transcription factor MEF/ELF4 regulates the quiescence of primitive hematopoietic cells. *Cancer Cell*, Mar; 9(3):175-87, 2006.
4. **Liu Y**, Hedvat CV, Mao S, Zhu XH, Yao J, Ngu yen H, Koff A, and Nimer SD. The ETS protein MEF is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCFSkp2. *Mol Cell Biol,* Apr; 26(8):3114-23, 2006. PMCID: PMC1446966
5. Yao JJ, **Liu Y**, Lacorazza HD, Soslow RA, Scandura JM, Mimer SD, and Hedvat CV. Tumor promoting properties of the ETS protein MEF in ovarian cancer. *Ocogene,* Jun 7; 26(27):4032-7, 2007.
6. Morales M, **Liu Y**, Laiakis EC, Morgan WF, Miner SD, and Petrini JH. DNA damage singaling in hematopoietic cells: a role for Mre11 complex repair of topoisomerase lesions. *Cancer Res*, Apr 1; 68(7):2186-93, 2008. PMCID: PMC2996041
7. **Liu Y**, Elf SE, Miyata Y, Sashida G, Liu Y, Huang G, Di Giandomenico S, Lee JM, Deblasio A, Menendez S, Antipin J, Reva B, Koff A, and Nimer SD. p53 regulates hematopoietic stem cell quiescence. *Cell Stem Cell*, Jan 9; 4(1):37-48, 2009. PMCID: PMC2839936
8. Lin HK, Wang G, Chen Z, Teruya-Feldstein J, **Liu Y,** Chan CH, Yang WL, Erdjument-Bromage H, Nakayama KI, Nimer S, Tempst P, and Pandolfi PP. Phosphorylation-dependent regulation of cytosolic localization and oncogenic function of Skp2 by Akt/PKB. *Nat Cell Biol*, Apr; 11(4):420-32, 2009. PMCID: PMC2830812
9. Sashida G, **Liu Y**, Elf S, Miyata Y, Ohyashiki K, Izumi M, Menendez S, and Nimer SD. ELF4/MEF activates MDM2 expression and blocks oncogene-induced p16 activation to promote transformation. *Mol Cell Biol*, Jul; 29(13):3687-99, 2009. PMCID: PMC2698769

10. **Liu Y**, Elf SE, Asai T, Miyata Y, Liu Y, Sashida G, Huang G, Di Giandomenico S, Koff A, and Nimer SD. The p53 tumor suppressor protein is a critical regulator of hematopoietic stem cell behavior. *Cell Cycle*, Oct1; 8(19):3120-4, 2009. PMICID: In progress.
11. **Liu Y**, Hoya-Arias R, and Nimer SD. The role of p53 in limiting somatic cell reprogramming. *Cell Res*, Nov; 19(11):1227-8, 2009.
12. **Liu Y**, Asai T, and Nimer SD. Myelodysplasia: battle in the bone marrow. *Nat Med*, Jan; 16(1):30-2, 2010.
13. MiyataY, **Liu Y**, jankovic V, Sashida G, Lee JM, Shieh JH, Naoe T, Moore M, and Nimer SD. Cyclin C regulates human hematopoietic stem/progenitor cell quiescence. *Stem Cells*, Feb; 28(2):308-17, 2010. PMCID: PMC3144254
14. Sashida G, Bazzoli E, Menendex S, **Liu Y**, and Nimer SD. The oncogenic role of the ETS transcription factors MEF and ERG. *Cell Cycle*, Sep 1; 9(17):3457-9, 2010. PMCID: PMC3230474
15. Sashida G, Bae N, Di Giandomenico S, Asai T, Gurvich N, Bazzoli E, **Liu Y**, Huang G, Zhao X, Menendez S, Nimer SD. (2011). The Mef/Elf4 transcription factor fine tunes the DNA damage response. *Cancer Research*, July 15; 71(14): 4857-65, 2011. PMCID: PMC4073677
16. Asai T, **Liu Y**, Bae N, and Nimer SD. The p53 tumor suppressor protein regulates hematopoietic stem cell fate. *J Cell Physiol*, Sep; 226(9):2215-21, 2011. PMCID: PMC3575444.
17. Huang G, Zhao X, Wang L, Elf S, Xu H, Zhao X, Sashida G, **Liu Y**, Lee J, Menendez S, Yang Y, Yan Y, Zhang P, Tenen DG, Osato M, Hsieh J, and Nimer SD. The ability of MLL to bind RUNX1 and methylate H3K4 at PU.1 regulatory regions is impaired by MDS/AML-associated RUNX1/AML1 mutations. *Blood,* Dec 15;118(25):6544-52*,* 2011. PMCID: PMC3242717
18. Hu P, Carlesso N, Xu M, **Liu Y**, Nebreda AR, Takemoto C, and Kapur R. Genetic evidence for critical roles of P38α in regulating mast cell differentiation and chemotaxis through distinct mechanisms*. J. Biol. Chem,* June 8*;*287(24):20258-69, 2012. PMCID: PMC3370208
19. Asai T, **Liu Y**, Di Giandomenico S, Bae N, Xu H, Nadiaye-Lobry D, Deblasio A, Menendez S, Antipin J, Reva B, Wevrick R, and Nimer SD. Necdin, a p53 target gene, regulates hematopoietic stem/progenitor cell quiescence and response to genotoxic stress. *Blood*, Aug 23;120(8):1601-12, 2012. PMC3429304. **Co-first author**.
20. Hu P, Nebreda AR, **Liu Y**, Carlesso N, Kaplan M, Kapur R. P38α negatively regulates T helper type 2 responses by orchestrating multiple TCR-associated signals. Journal of Biological Chemistry, Sep 28; 287(40): 33215-26, 2012. PMCID: PMC3460427
21. Vemula S, Shi J, Mali RS, Ma P, **Liu Y**, Hanneman P, Koehler KR, Hashino E, Wei L, and Kapur R. ROCK1 functions as a critical regulator of stress erythropoiesis and survival by regulating p53. *Blood*, Oct 4; 120(14):2868-78, 2012. PMCID: PMC3466968
22. **Liu Y***, Liu F, Yu H, Zhao X, Sahsida G, Deblasio A, Chen Z, Lin HK, Di Giandomenico S, Elf SE, Yang YY, Miyata Y, Huang G, Menendez S, Mellinghoff I, Pandolfi PP, Hedvat CV and Nimer SD. Akt Phosphorylates the Transcriptional Repressor Bmi1 to Block Its Association with Tumor Suppressing *Ink4a-Arf* locus. *Science Signaling*, October 23; 5, ra77, 2012. PMCID: PMC3784651. **Corresponding author**.
23. **Liu Y***, Yu H, Nimer SD. PI3K-Akt pathway regulates polycomb group protein and stem cell maintenance. *Cell Cycle.* 12(2):199-200, 2013. PMCID: PMC3757235. **Corresponding author**.
24. Asai T, **Liu Y** and Nimer SD. Necdin, a p53 target, in normal and cancer stem cells. *Oncotarget.* 4(6):806-7, 2013. PMCID: PMC3757235
25. Sabelli PA, **Liu Y**, Dante RA, Lizarraga LE, Nguyen HN, Brown SW, Klingler JP, Yu J, LaBrant E, Layton TM, Feldman and Larkins BA. Control of Cell Proliferation, Endoreduplication, Cell Size and Cell Death by the Retinoblastoma-Related Pathway in Maize Endosperm. *Proc Natl Acad Sci U S A.* 110(19):E1827-36, 2013. PMCID: PMC3651506

26. Vu LP, Perna F, Wang L, Voza F, Figueroa ME, Tempst P, Erdjument-Bromage H, Gao R, Chen S, Paietta E, Deblasio T, Melnick A, **Liu Y**, Zhao X and Nimer SD. PRMT4 blocks myeloid differentiation by assembling a methyl-RUNX1-dependent repressor complex. *Cell Reports.* 5(6):1625-38, 2013. PMCID: PMC4073674
27. Nimer SD, **Liu Y***. ELF4 [E74-like factor 4 (ets domain transcription factor)]. *Atlas Genet Cytogenet Oncol Haematol.* 18(7):511-515, 2014 (**Corresponding author**).
28. Kobayashi M, Bai Y, Dong Y, Yu H, Chen S, Gao R, Zhang L, Yoder MC, Kapur R, Zhang ZY and **Liu Y***. PRL2/PTP4A2 phosphatase is important for hematopoietic stem cell self-renewal. *Stem Cells*. 32(7):1956-67, 2014. PMCID: PMC4063874. **Corresponding author**.
29. Kobayashi M, Shelly WC, Seo W, Vemula S, **Liu Y**, Kapur R, Taniuchi I, Yoshimoto M. Functional B-1 progenitor cells are present in the hematopoietic stem cell deficient embryo and depend on Cbfβ for their development. *Proc N atl Acad Sci U S A.* 111(33):12151-6, 2014. PMCID: PMC4143017
30. Gao R and **Liu Y***. Protein synthesis: more than a house-keeping function in hematopoietic stem cells. *Journal of Stem Cell Research and Transplantation*. 2014; 1(1):2. **Corresponding author**.
31. Kobayashi M, Chen S, Gao R, Bai Y, Zhang ZY, and **Liu Y***. Phosphatase of regenerating liver in hematopoietic stem cell self-renewal and hematological malignancies. *Cell Cycle,* 2014;13(18): 2827-2835. PMCID: *In progress.* **Corresponding author**.
32. Gao R, Chen S, Kobayashi M, Yu H, Young SK, Soltis A, Zhang Y, Wan Y, Vemula S, Fraenkel E, Cantor A, Xu Y, Yoder MC, Wek R, Kapur R, Ellis S, Zhu X and **Liu Y***. Bmi1 promotes erythropoiesis through regulating ribosome biogenesis. *Stem Cells*. 2015 Mar;33(3):925-38. PMCID:PMC4380436. **Corresponding author**.
33. Chatterjee A, Ghosh J, Ramdas B, Mali RS, Martin H, Kobayashi M, Vemula S, Canela VH, Waskow ER, Visconte V, Tiu RV, Smith CC, Shah N, Bunting KD, Boswell HS, **Liu Y*†**, Chan RJ, Kapur R. Regulation of Stat5 by FAK and PAK1 in Oncogenic FLT3- and KIT-Driven Leukemogenesis. *Cell Reports*. 2014 Nov 20;9(4):1333-1348. PMCID:PMC4380442.
34. Chen S, Gao R, Kobayashi M, Yoder M and **Liu Y***. Pharmacological inhibition of AKT activity in human CD34+ cells enhances their engraftment in immunodeficient mice. *Experimental Hematology* (Submitted, 2015). **Corresponding author**.
35. Kobayashi M, Bai Y, Yoshimoto M, Chen S, Gao R, Zhang L, Zhang ZY and **Liu Y***. PRL2/PTP4A2 phosphatase is important for T-cell development. *Journal of Clinical Investigation* (Submitted, 2015). **Corresponding author**.
36. Zhang J, Kong G, Lu L, Zhang X, Ranheim EA, Liu Y, Rajagopalan A, Wang J, Gao X, Chang YI, Zhou Y, Yang D, Bhatnagar B, Lucas DM, **Liu Y**, Bresnick E, Zhong X, Zhang J. *p53*$^{-/-}$ synergizes with enhanced *Nras*^{G12D} signaling in acute myeloid leukemia. Blood (Submitted, 2015).
37. Chen S, Yu H, Kobayashi M, Gao R, Boswell HS, Xu Y and **Liu Y***. Gain of function mutant p53 enhances hematopoietic stem cell self-renewal. *Cell Stem Cell* (In preparation, 2015). **Corresponding author**.
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FEDERAL FINANCIAL REPORT

(Follow form instructions)

1. Federal Agency and Organizational Element to Which Report is Submitted	2. Federal Grant or Other Identifying Number Assigned by Federal Agency (To report multiple grants, use FFR Attachment)	Page of
Army Medical Research And Material Command	**W81XWH-13-1-0187**	1 1 pages

3. Recipient Organization (Name and complete address including Zip code)

Indiana University, Office of Research Administration, 509 East Third Street, Bloomington, IN 47401

4a. DUNS Number	4b. EIN	5. Recipient Account Number or Identifying Number (To report multiple grants, use FFR Attachment)	6. Report Type	7. Basis of Accounting
62-638-0133	**35-6001673**	**43-860-48**	☐ Quarterly ☐ Semi-yearly ☐ Annual ☑ Final	☑ Cash ☐ Accrual

8. Project/Grant Period (Month, Day, Year)		9. Reporting Period End Date (Month, Day, Year)
From: **07/01/13**	To: **06/30/15**	**06/30/15**

10. **Transactions**	Cumulative
(Use lines a-c for single or multiple grant reporting)	
Federal Cash (To report multiple grants, also use FFR Attachment):	
a. Cash Receipts	374,400.00
b. Cash Disbursements	374,400.00
c. Cash on Hand (line a minus b)	-
(Use lines d-o for single grant reporting)	
Federal Expenditures and Unobligated Balance:	
d. Total Federal funds authorized	374,400.00
e. Federal share of expenditures	374,400.00
f. Federal share of unliquidated obligations	-
g. Total Federal share (sum of lines e and f)	374,400.00
h. Unobligated balance of Federal funds (line d minus g)	-
Recipient Share:	
i. Total recipient share required	-
j. Recipient share of expenditures	-
k. Remaining recipient share to be provided (line i minus j)	-
Program Income:	
l. Total Federal program income earned	-
m. Program income expended in accordance with the deduction alternative	-
n. Program income expended in accordance with the addition alternative	-
o. Unexpended program income (line l minus line m or line n)	-

11. Indirect Expense	a. Type	b. Rate	c. Period From	Period To	d. Base	e. Amount Charged	f. Federal Share
	Predetermined	**56.0%**	07/01/13	06/30/15	$239,999.99	134,400.00	134,400.00
				g. Totals:	239,999.99	134,400.00	134,400.00

12. Remarks: Attach any explanations deemed necessary or information required by Federal sponsoring agency in compliance with governing legislation:

13. Certification: By signing this report, I certify to the best of my knowledge and belief that the report is true, complete, and accurate, and the expenditures, disbursements and cash receipts are for the purposes and intent set forth in the award documents. I am aware that any false, fictitious, or fraudulent information may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

a. Typed or Printed Name and Title of Authorized Certifying Official	c. Telephone (Area code, number, and extension)
KC Forthofer, Financial Analyst	**(812) 856-2317**
b. Signature of Authorized Certifying Official	d. Email Address
	kcfort@iu.edu
	e. Date Report Submitted (Month, Day, Year)
	August 24, 2015
	14. Agency use only:

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OMB Approval Number: 0348-0061
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